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Yan Xu

▶ **To cite this version:**

Yan Xu. Characterization of Hepatitis C Virus interaction with heparan sulfate proteoglycans. Human health and pathology. Université du Droit et de la Santé - Lille II, 2014. English. NNT : 2014LIL2S021 . tel-01201824

HAL Id: tel-01201824
https://theses.hal.science/tel-01201824

Submitted on 18 Sep 2015

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THÈSE DE DOCTORAT DE L'UNIVERSITÉ LILLE II
École Doctorale Biologie –Santé de Lille

Pour l'obtention du grade de :
Docteur de L'UNIVERSITÉ LILLE II

Thèse soutenue le 16 Septembre 2014

Caractérisation des interactions du Virus de l'Hépatite C avec les protéoglycanes à héparane sulfate

Characterization of Hepatitis C Virus interaction with heparan sulfate proteoglycans

Centre d'Infection & d'Immunité de Lille (CIIL), Inserm U1019, CNRS UMR 8204,
Institut Pasteur de Lille, Équipe Virologie Moléculaire & Cellulaire de l'Hépatite C

Directeur de Thèse: **Dr Jean Dubuisson**

Présentée et soutenue publiquement par:

Yan XU

Membres du Jury:

Président	**Pr. Didier HOBER**
Rapporteur	**Pr. Sandrine CASTELAIN**
Rapporteur	**Pr. Philip MEULEMAN**
Examinateur	**Dr. Sandrine BELOUZARD**
Examinateur	**Dr. François HELLE**
Directeur de thèse	**Dr. Jean DUBUISSON**

To my parents and my dearest friend Manuel

Dreams

Hold fast to dreams
For if dreams die
Life is a broken-winged bird
That cannot fly.

Hold fast to dreams
For when dreams go
Life is a barren field
Frozen with snow.

-Langston Hughes

ACKNOWLEDGMENTS

At the beginning, I would like to thank all the members of jury: professors **Didier Hober**, **Philip Meuleman** and **Sandrine Castelain**, and doctors **François Helle** and **Sandrine Belouzard**, and my supervisor Dr. **Jean Dubuisson**. Thanks to all of you for the attendance and participation in my thesis defence.

Then, I would like to give my sincere gratitude again to my supervisor Dr. **Jean Dubuisson**. Thanks for your initial help and the precious opportunity given to me so that I could do my PhD study in your laboratory. Thanks very much for your patience and great kindness to me all along the way. Also, thanks for your scientific guidance and putting in. Next, I would like to sincerely acknowledge my “petit chef ” Dr. **Sandrine Belouzard**. Thanks for your generous access to me in the whole process of my project. With you, I learned a lot, from basic experimental techinque to critical thinking on the results. I believe that your strict attitude and whole-hearted dedication into scientific research, as well as your confident decision-making manner have already infected me, and from which I can benefit for my future career. Hereafter, I also thank other scientists including Drs **Guangxiang Luo, Mansun Law, Steven Foung** and **Jane A. McKeating**, for your kindly providing us the antibodies that are very important for this work. And then thanks to Dr. **Karin Séron**, for your technical assistance which contributed a lot to this work. Certainly, thanks very much for the scientific input from our collaborators Dr. **Allain Fabrice** and **Pierre Martinez.**

I am very grateful to M. **Sophana Ung**. Thanks for your scientific and plenty of non-scientific support. Also, for all our talking and telling me “this world is not black and white”. Another impressing meet- Pr. **Yves Rouillé**. You are so wise and farsighted. Your passion to research is obvious. You are always in the state of thinking. Very nice to meet you and talk with you, and also thank you for your professional contribution to our work.

Then, many thanks to people in the lab. including **Laurence, Muriel, Anne, Rayan, Juliette, Allison**, **Marie-Emmanuelle, Nathalie, Claire, Adeline, Lucie, Nana, Lison, Laura,** and a pair of old mates: **Czes** and **André**. Also thanks to previous members : **Sohail, Ahmed, Julie, PYD, Noémie** and **Thibaut**. And, to my French teacher **Anaïs** who makes me like French Language more and more. In a word, thanks to all the people ‘‘J'ai rencontré d’ici’’, thanks for your help and those time we passed together.

At last, I want to thank friends here **Fan, Jia, Odile** and **Julie**. Thanks for your help, and the time with you that enriched my days in France. Also, thanks to my foundation support from CSC and my family backing, which together made my short ‘‘life journey’’ in France possible, then I could travel around Europe a bit, know China better and love her more.

RÉSUMÉ

L’entrée du virus de l’hépatite C (VHC) dans les hépatocytes est un événement multi-étapes complexe, impliquant un certain nombre de facteurs cellulaires. Elle est initiée par la fixation des particules virales sur des structures d’héparanes sulfates (HS) présentes à la surface de l’hépatocyte. Cette étape initiale reste cependant peu comprise. En effet, en raison de l’interaction de la particule virale du VHC avec des lipoprotéines, la contribution exacte des différents composants du virion à cette interaction reste controversée. Au cours de cette thèse, nous avons étudié le rôle potentiel de protéines d'enveloppe du VHC et de l'apolipoprotéine E dans l'étape de liaison aux HS. Nous avons d’abord montré que la délétion de la région hypervariable 1 (HVR1), une région précédemment proposée pour participer à l’interaction avec les HS, n'avait aucun effet sur la liaison du virion aux HS, indiquant que cette région n'est pas impliquée dans cette interaction. Nous avons également utilisé des anticorps monoclonaux neutralisants reconnaissant différentes régions des glycoprotéines d'enveloppe du VHC dans un test de compétition utilisant des billes d’agarose couplées à l’héparine, un homologue structural des HS, pour précipiter le virus. Bien que les glycoprotéines d’enveloppe du VHC dissociées de la particule virale interagissaient avec l'héparine, aucun de ces anticorps n’était capable d'interférer avec l'interaction entre la particule virale et l’héparine, suggérant fortement que les glycoprotéines d'enveloppe du VHC présente à la surface des virions ne sont pas accessibles pour interagir avec les HS. En revanche, nos résultats d’études cinétiques, d’interaction avec l’héparine ainsi que les expériences d'inhibition avec des anticorps anti-apolipoprotéine E indiquent que cette apolipoprotéine joue un rôle majeur dans l'interaction initiale entre le VHC et les HS. Enfin, la caractérisation des déterminants structuraux des HS nécessaires à l'infection par le VHC, à l’aide d’ARN interférents ciblant des enzymes impliquées dans la voie de biosynthèse des HS et par compétition avec des héparines modifiées, indique que la N-sulfatation et la 6-O-sulfatation sont nécessaires pour l’initiation de l'infection par le VHC. Par contre la 2-O-sulfatation n’est pas indispensable pour l’étape d’entrée cellulaire du VHC. Enfin, nous avons également montré que la taille minimale des oligosaccharides d’HS requise pour l'infection par le VHC est un decasaccharide. En conclusion, l’ensemble de ces données indique que le VHC détourne l'apolipoprotéine E pour initier son interaction avec des structures d’HS spécifiques.

RÉSUMÉ LONG

L' infection par le virus de l'hépatite C **(VHC)** demeure un problème majeur de santé publique, qui conduit fréquemment à des maladies chroniques du foie pouvant conduire à une cirrhose, une insuffisance hépatique et un carcinome hépatocellulaire (CHC) (Saito *et al*., 1990). Au niveau modial, environ 170 millions de personnes (3% de la population mondiale) sont actuellement infectées par ce virus. Plus de 350 000 personnes meurent de maladies liées au VHC chaque année. De plus, on compte près de 4 millions de nouvelles infections par an (Mohd *et al.*, 2013). Les hépatites virales, comprenant essentiellement l'hépatite C et l'hépatite B, représentent environ 75% de tous les cas de maladie du foie dans le monde entier (Ananthakrishnan *et al*., 2006), et le cancer du foie majoritairement induit par ces infections virales représente le troisième cancer le plus frequent au monde.

L'infection par le VHC résulte en une hépatite aiguë qui peut se résoudre spontanément sans symptôme particulier de maladie hépatique excepté une jaunisse dans seulement 20% des cas (Loomba *et al*., 2011). Pour 80% des individus infectés, l'infection conduit à une hépatite chronique: 15-30% des cas évoluent vers une cirrhose alors que 1-3% d'entre eux nécessitent une greffe de foie ou risquent de mourir d'une cirrhose ou d'un cancer du foie annuellement. En général, une évolution de la maladie typique de l'infection vers des maladies hépatiques symptomatiques se déroule dans un laps de temps de 20-30 ans. Beaucoup d'infections chroniques par le VHC ne donnent pas lieu à des symptômes au cours de cette période, bien que certaines personnes éprouvent de la fatigue, de la dépression ou d'autres manifestations extra-hépatiques (Galossi *et al*., 2007). Cela est la principale raison pour laquelle la plupart des personnes infectées ne sont pas diagnostiquées jusqu'à ce qu'ils développent des maladies graves du foie. Les facteurs qui favorisent la progression de l'hépatite chronique comprennent la consommation d'alcool, le sexe, l'âge au moment de l'infection et un déficit immunitaire (Poynard *et al*., 2007; Lauer & Walker, 2001; Chen & Morgan, 2006). Il est à noter que la co-infection avec le virus de l'immunodéficience humaine (VIH) et /ou le virus de l'hépatite B (VHB) sont des facteurs de risque importants pour la fibrose du foie (Benhamou *et al*., 1999; Raimondo *et al*., 2005). Particulièrement, la co-infection VHC-VHB peut augmenter le risque de CHC (Benvegnu *et al*., 1994; Donato *et al*., 1998). Par ailleurs, la charge virale et le génotype du VHC ne semblent pas affecter la vitesse de progression. Toutefois, des preuves

montrent que l'infection par le VHC de génotype 3 est associée à la progression accélérée de la fibrose (Bochud *et al*., 2009).

Le VHC se transmet principalement par l'exposition au sang contaminé, c'est pourquoi l'hépatite virale C, qui est provoquée par le VHC, a été tout d'abord appelé hépatite non-A, non-B post-transfusionnelle (Alter, 1978; Kuo *et al*., 1989). De nos jours, les utilisateurs de drogues injectables sont fréquemment infectés par le VHC (Khalsa & Elkashef, 2010; Hagan & Des Jarlais, 2000; Liu *et al*., 2008; Xia *et al*., 2008). Un autre mode de transmission occasionnel s'effectue par contact sexuel. Bien que la transmission par voie sexuelle concerne une minorité de cas, au cours des dernières années l'hépatite aiguë C est devenu un problème chez les hommes homosexuels infectés par le VIH (Lopez-Diguez *et al*., 2011).

Le traitement de l'infection par le VHC a évolué fortement au cours des 20 dernières années. D'abord basé sur l'utilisation d'interféron alpha, il a ensuite évolué vers une combinaison thérapeutique incluant aussi la ribavirine. De plus, au début des années 2000, une modification de l'interféron par un ajout de polyéthylèneglycol (PEG) a permis d'améliorer les paramètres pharmacocinétique de cette molécule. Pendant une dizaine d'années, le traitement standard de l'hépatite C chronique a été la combinaison de l'interféron alpha pégylé (PEG-IFN) et de la ribavirine (RBV). Récemment, un inhibiteur de la protéase du VHC (soit telaprevir ou boceprevir) a été ajouté à la thérapie standard pour le traitement de l'infection par le VHC de génotype 1 (Bacon *et al*., 2011; Jacobson *et al*., 2011). Plus récemment encore, d'autres inhibiteurs spécifiques du VHC ont montré des résultats cliniques spectaculaires et le traitement de cette infection virale est en train de subir une révolution avec notamment l'utilisation du sofosbuvir, un inhibiteur de la polymérase virale.

Le VHC est un petit virus enveloppé d'ARN de sens positif, il appartient au genre Hepacivirus dans la famille des *Flaviviridae*. Sur la base des analyses phylogénétiques de la région core/E1 et NS5 du génome viral, le VHC est classé en 6 génotypes principaux, numérotés du génotype 1 au génotype 6. Ces informations concernant le génotype revêtent une importance considérable, car elles offrent une référence essentielle dans le traitement médical du VHC. En effet, les décisions concernant la durée du traitement, la dose de ribavirine et si un inhibiteur de la protéase du VHC est ajouté à la thérapie standard, sont dépendantes du génotype (Mangia & Mottola, 2012; Slavenburg *et al*., 2009; Kwo, 2011).

Le génome du VHC est un ARN simple brin de 9.6kb composé des régions 5' et 3' non-traduites (NTRs) et d'un grand cadre de lecture ouvert (ORF), qui code une polyprotéine précurseur de ~ 3000 acides aminés (Choo *et al*., 1991). Cette polyprotéine est co- et post-traductionnellement clivée en au moins 10 protéines virales dont 3 protéines structurales (la protéine de capside ou protéine core et les protéines d'enveloppe E1 et E2) et 7 protéines non structurales (p7, NS2, NS3, NS4A, NS4B, NS5A et NS5B) (Fig.5) (Moradpour *et al*., 2007). Les protéines non structurales sont essentielles à la réplication virale, alors que les protéines structurales du VHC forment le virion (Moradpour *et al*., 2007; Bartenschlager, 2013).

En particulier, ***les protéines d'enveloppe du VHC E1 et E2*** sont les composants essentiels de l'enveloppe virale, étant exposées sur le virion, elles jouent un rôle majeur dans l'entrée du VHC et l'assemblage du virion. E1 et E2 sont clivées à partir de la polyprotéine par une signal-peptidase du réticulum endoplasmique. Ce sont des protéines transmembranaires de type I, contenant un grand domaine extracellulaire N-terminal et un petit domaine transmembranaire hydrophobe C-terminal. Les ectodomaines de E1 et E2 sont hautement glycosylées, portant respectivement jusqu'à 5 et 11 sites de N-glycosylation (Fig.6) (Lavie *et al*., 2007). Ces N-glycanes sont impliqués dans le repliement des glycoprotéines d'enveloppe, par association avec des protéines chaperonnes du réticulum endoplasmique (Dubuisson & Rice, 1996; Choukhi *et al*., 1998; Lavie *et al*., 2007). Récemment, il a été montré que plusieurs glycanes sur E2 masquent la reconnaissance d'épitopes neutralisants (Helle *et al*., 2007; Helle *et al*., 2010). Ce type d'évasion immunitaire aidée par le bouclier de glycanes des glycoprotéines d'enveloppe virale est également observé pour d'autres virus tels que le VIH. Par conséquent, les glycanes apparaîssent intéressants comme nouvelle cible potentielle pour le développement de molécules antivirales (Feld *et al*., 2014; McLellan *et al*., 2011).

Les domaines transmembranaires de E1 et E2 contiennent les signaux de rétention dans le réticulum endoplasmique, ils sont impliqués dans l'hétérodimérisation E1E2 et ils contiennent des séquences signal qui servent à la translocation de l'ectodomaine de la protéine située en aval dans la polyprotéine (Cocquerel *et al*., 1998; Cocquerel *et al*., 1999). L'accumulation intracellulaire de protéines d'enveloppe du VHC dans le réticulum endoplasmique a été montré comme étant indispensable pour la formation des hétérodimères E1E2 et l'assemblage des protéines de l'enveloppe (Cocquerel *et al*., 2000; Op De Beeck *et al*., 2004). Il est à noter que les formes intracellulaires de E1 et E2 s'assemblent comme hétérodimères non covalents. Les formes extracellulaires, qui sont incorporées dans des virions infectieux, sont quant à

elles formées de gros complexes covalents stabilisées par des ponts disulfures (Vieyres *et al*., 2010; Op De Beeck *et al*., 2000; Krey *et al*., 2010).

Comme pour les autres virus à ARN brin positif, ***le cycle viral du VHC*** est entièrement cytoplasmique. Il commence avec l'entrée du VHC dans les hépatocytes par l'endocytose. Après la décapsidation de la particule virale, l'ARN génomique est libéré dans le cytoplasme. Ce dernier est instantanément traduit, grâce à un site d'entrée ribosomal interne (IRES) du VHC, au niveau du réticulum endoplasmique, en un précurseur polyprotéique. Ce précurseur protéique est co- ou post-traductionnellement clivé par des proteases cellulaires et virales en protéines structurales et non structurales matures. Au sein de radeaux lipidiques resistants aux détergents, les protéines virales NS3-NS5 forment des complexes de réplication où NS5B (ARN polymérases dépendante de l'ARN) dirige la synthèse des ARN nouveaux en utilisant des matrices d'ARN complémentaires du VHC (Aizaki *et al*., 2003; Shi *et al*., 2003). L'ARN brin positif nouvellement synthétisé est encapsidé par la protéine de capside mature qui homodimérise et est transporté vers des gouttelettes de lipides (LDs). Dans un environnement proche des LD, les particules virales s'assemblent par recrutement des protéines d'enveloppe du VHC suivi du bourgeonnent dans la lumière du réticulum endoplasmique. Dans la voie de sécrétion cellulaire, les particules virales subissent une maturation en interagissant avec les lipoprotéines de très faible densité. Ensuite, les particules virales matures sortent de la cellule infectée. La protéine p7 semble jouer un rôle important pour empêcher la fusion précoce de la particule au sein de la voie de sécrétion (Fig.7) (Wozniak *et al*., 2013).

Des études approfondies ont montré que ***le cycle viral du VHC est étroitement lié au métabolisme des lipides des cellules hépatiques*** (Ye *et al*., 2003; Huang *et al*., 2007; Ye, 2007; Herker & Ott, 2001). L'interaction entre le VHC et les lipides cellulaires commence peu de temps après la synthèse des protéines virales dans les cellules infectées. Il a été montré que la réplication de l'ARN du VHC dépend de lipides et notamment du cholestérol cellulaire (Kapadia *et al*., 2003; Ye *et al*., 2003; Kapadia & Chisari, 2005). Aussi, il a été constaté que les vésicules membranaires dans lesquelles le VHC se réplique, contenant le complexe de replication du VHC, sont hautement enrichies en protéines nécessaires pour l'assemblage des VLDL, y compris l'apolipoprotéine B (apoB), l'apoE et la protéine microsomale de transfert des triglycérides (MTP) (Huang *et al*., 2007). Les hépatocytes jouent un rôle crucial dans la régulation du métabolisme du cholestérol chez les mammifères, par l'exportation du cholestérol avec des triglycerides, conduisant à la formation des VLDL. Il a été montré que

dans les cellules d'hépatome qui produisent le VHC infectieux, la production du VHC est diminuée après traitement à l'aide d'agents qui bloquent l'assemblage des VLDL tels qu'un inhibiteur de la MTP et un siRNA dirigé contre l'apoB. Ces données suggèrent l'importance d'une voie fonctionnelle de sécrétion des VLDL pour la production de particules infectieuses du VHC (Huang *et al*., 2007; Gastaminza *et al*., 2008). Cette interconnexion entre la morphogenèse du VHC et la biogenèse des VLDL conduit à la production de virions matures enrichis en cholestérol et sphingolipides. De plus, il a été constaté que le cholestérol et les sphingolipides associés au virion sont importants pour la maturation et l'infectiosité des virions (Aizaki *et al*., 2008).

Enfin, le lien étroit entre le cycle de viral du VHC et le métabolisme cellulaire des lipides conduit à une caractéristique unique des ***virions du VHC***. En effet, en plus des proteines structurales virales, les particules du VHC sont aussi associées à des lipoprotéines, et contiennent des apolipoprotéines telles que apoE apoB, apoCI, CII et CIII (André *et al*., 2002; Nielsen *et al*., 2004; Diaz *et al*., 2006; Maillard *et al*., 2006; Chang *et al*., 2007; Meunier *et al*., 2008). De plus, chez les patients, des particules de VHC circulant dans le sang présentent une hétérogénéité de densité. André *et al* ont quantifié l'ARN du VHC à partir de sérums de patients dans les fractions de faible densité correspondant à des fractions de lipoprotéines de très basse densité, de lipoprotéines de densité intermédiaire et lipoprotéines de basse densité (VLDL, IDL, LDL). Il a été trouvé que tous les patients avaient de l'ARN du VHC dans au moins une de ces trois fractions. Ces particules contenant de l'ARN du VHC purifiées ont été appelés ***lipo-viro-particules*** **purifiées (*LVPs*)** (André *et al*., 2002).

Une caractérisation biochimique comparative des LVP purifiées et des VLDL a montré que les LVP purifiées contenaient plus de triglycéride par molécule ApoB que des lipoprotéines isolées de fractions équivalentes après séparation sur gradient de densité. Cette différence dans la composition lipidique suggère que les LVP ne sont pas seulement des virions du VHC interagissant avec des lipoprotéines. En microscopie électronique, les LVP purifiées semblent être composées de grandes structures sphériques avec un diamètre moyen de 100 nm (Thomssen *et al*., 1992; Thomssen *et al*., 1993) Après délipidation, les LVP purifiées sont apparues sous forme de particules de capside dont les plus grandes structures sont de 30-35nm de diamètre (André *et al*., 2002). De façon surprenante, il a également été constaté que chez les patients chroniquement infectés par le VHC, on retrouvait une proportion importante de LVP dépourvues de nucléocapsides, mais contenant des protéines d'enveloppe et des

lipoprotéines, formant ainsi des particules sous-virales (eLVPs) (Scholtes *et al*., 2012). Ceci est cohérent avec l'observation selon laquelle la densité des particules de VHC dans le plasma de patients était très variable (Thomssen *et al*., 1992; Scholtes *et al*., 2012).

Etant donné leur hétérogénéité, les particules de VHC ne sont pas parfaitement caractérisées. Il est cependant clair que l'apoE est un élément essentiel du viron VHC présent à la surface de la particule. L'analyse des apolipoprotéines contenues dans du virus produit en culture cellulaire et purifié a montré que chacune des particules contient près de 300 molécules d' apoE à sa surface, ce qui suggère un enrichissement remarquable en molécules d'apoE sur le virion VHC (Merz *et al*., 2010). Une caractérisation en microscopie électronique plus récente a également montré la présence des apolipoprotéines apoB et apoA-I à la surface du virion, en plus de l'apoE (Catanese *et al*., 2013b). De plus, il semble que les glycoprotéines virales soient peu accessibles aux anticorps spécifiques (Catanese *et al*., 2013b).

L'entrée du VHC dans les hépatocytes est un événement multi-étapes complexe, impliquant un certain nombre de facteurs cellulaires. Elle est initiée par la fixation des particules virales à la surface des cellules hôtes. Les protéoglycanes à héparane sulfate (HSPG) et le récepteur des lipoprotéines de faible densité (LDLr), ont été proposés comme les facteurs d'attachement responsables de l'interaction initiale entre le virion VHC et la surface cellulaire (Agnello *et al*., 1999; Monazahian *et al*., 1999; Germi *et al*., 2002; Barth *et al*., 2003). Ensuite, une série de facteurs cellulaires spécifiques entrent en jeu. Les principaux facteurs d'entrée identifiés sont la tétraspanine CD81, le récepteur Scavenger B1 (SRB1) et les protéines de jonctions serrées, Claudine-1 (CLDN1) et Occludine (OCLN) (Lindenbach & Rice, 2013). Il semble que l'association entre le virion VHC et SRB1, par l'intermédiaire de la région HVR1 de E2, soit importante pour l'interaction entre la glycoprotéine E2 et CD81. Une interaction entre CD81 et CLDN1 est également essentielle pour assurer l'entrée du VHC (Bartosch *et al*., 2003b; Harris *et al*., 2008; Stamataki *et al*., 2008; Harris *et al*., 2010). La protéine OCLN semble intervenir plus tardivement dans l'entrée virale et son rôle exact reste à préciser (Sourisseau *et al*., 2013). Finalement, les particules de VHC entrent dans l'hépatocyte par endocytose médiée par la clathrine (Blanchard *et al*., 20006). Le pH acide des endosomes précoces induit la fusion du VHC avec la membrane de ce compartiment, conduisant à la décapsidation de la particule virale et au transfert de l'ARN génomique du VHC dans le cytoplasme (Meertens *et al*., 2006). En plus de CD81, SRB1, CLDN1 et OCLN, d'autres facteurs cellulaires impliqués dans l'entrée virale ont récemment été identifiés. Il s'agit

essentiellement du récepteur au facteur de croissance épidermique (EGFR) (Lupberger *et al*., 2011), du récepteur de ephrin A2 (Lupberger *et al*., 2011), du récepteur d'absorption du cholestérol Niemann-Pick C1-like 1 (NPC1L1) (Sainz *et al*., 2012) et du récepteur de la transferrine 1 (TfR1) (Martin & Uprichard, 2013). Il est important de noter que le récepteur EGFR est impliqué dans l'entrée du VHC en contrôlant les interactions entre CD81 et CLDN1(Lupberger *et al*., 2011). De plus, il a également été montré que la GTPase HRas joue un rôle essentiel dans la transduction de signal nécessaire pour le trafic membranaire de CD81 à la surface cellulaire, permettant l'assemblage de complexes de récepteurs-tétraspanines (Zona *et al*., 2014) (Fig. 16) .

L'entrée du VHC dans les hépatocytes dépend des interactions directes entre les particules virales et les molécules exprimées à la surface cellulaire. Cependant, l'entrée peut également être indirectement contrôlée par des protéines intracellulaires/extracellulaires à travers l'interaction avec ces récepteurs/co-récepteurs. De façon intéressante, la protéine EWI-2wint, un partenaire de CD81, bloque l'entrée du VHC en inhibant l'interaction entre les protéines d'enveloppe du VHC et CD81 lorsqu'elle est exprimée dans des cellules hépatocytaires (Montpellier *et al*., 2011; Rocha-Perugini *et al*., 2008). Cette protéine EWI-2wint est exprimée dans de nombreuses lignées cellulaires non-permissives au VHC mais elle ne s'exprime pas dans des lignées cellulaires d'origine hépatocytaire (Rocha-Perugini *et al*., 2008). Ainsi, l'absence de ce facteur inhibiteur spécifique dans la cellule hôte semble être exploité par le VHC pour cibler spécifiquement les cellules du foie.

Comme pour de nombreux virus, les interactions virus-récepteurs chez le VHC permettent non seulement de déterminer le tropisme cellulaire, mais également la spécificité d'hôte. En effet, il a été montré que l'expression des récepteurs CD81, SRB1, CLDN1 et OCLN dans les hépatocytes de souris rendent les cellules murines permissive pour l'entrée du VHC (Ploss *et al*., 2009). Cependant, l'origine humaine de ces récepteurs est seulement nécessaire pour CD81 et OCLN. En outre, il est possible d'adapter le virus VHC à certains facteurs d'entrée murins, comme cela a été montré pour la tétraspanine murine CD81 (Bitzegeio *et al*., 2010)

Les glycoprotéines d'enveloppe du VHC jouent un rôle essentiel dans l'entrée virale et elles sont également les principales cibles des anticorps anti-VHC (Giang *et al*., 2012; Prenteo *et al*., 2011; Farci *et al*., 1996; Krey *et al*., 2013). E1 et E2 sont connues pour médier les interactions avec les récepteurs cellulaires, ainsi que la fusion entre l'enveloppe et les

membranes cellulaires suite à l'internalisation du virion. Le rôle de E1 dans le processus d'entrée du VHC n'est pas encore très clair, elle a été proposée comme jouant un rôle dans processus de fusion et une étude récente a suggéré son rôle en tant que modulateur pour la liaison aux récepteurs et la fusion membranaire du VHC (Lavillette *et al*., 2007; Douam *et al*., 2013; Wahid *et al*., 2013). Le rôle de la glycoprotéine E2 est cependant mieux caractérisé. Cette protéine interagit avec les récepteurs CD81 et SRB1 et elle est la cible principale des anticorps neutralisants (Pileri *et al*., 1998; Flint *et al*., 1999a; Scarselli *et al*., 2002; Bartosch *et al*., 2003; Zhang *et al*., 2004). Il est à noter que E2 contient une région hypervariable (HVR1), constituée d'un segment de 27 acides aminés situés à l'extrémité N-terminale de la protéine. Cette région a été originellement trouvée comme étant la séquence avec la plus grande hétérogénéité entre les isolats de HCV, et a été proposée comme un leurre immunologique (Ray *et al*., 1999; Farci *et al*., 2000; Penin *et al*., 2001). Il a ensuite été montré que cette région est responsable de l'interaction E2-SRB1 et est impliquée dans l'entrée virale (Scarselli *et al*., 2002; Bartosch *et al*., 2003).

Au cours de ma thèse, je me suis intéressée aux événements précoces de l'entrée cellulaire du VHC. ***L'objectif précis de mon travail était de caractériser l'interaction entre le VHC et les proteoglycanes à héparane sulfate (HSPG) impliqués dans l'attachement viral à la surface de l'hépatocyte.***

Les HSPG sont abondants dans la matrice de l'espace de Disse et à la surface des hépatocytes. Ils sont composés d'une partie protéique et de chaînes d'héparane sulfates (HS), qui sont des polysaccharides linéaires constitués d'une unité disaccharidique répétée comprenant un acide uronique et un dérivé de glucosamine avec différents motifs de sulfatation (Esko & Selleck, 2002). Il a d'abord été proposé que des particules de VHC isolées de patients interagissent avec les glycosaminoglycanes (Germi *et al*., 2002). Suite à cette observation, il a également été montré que la glycoprotéine d'envelope du VHC E2 ainsi que des glycoprotéines d'enveloppe isolées de virions purifiés, interagissent avec des HSPG, suggérant un contact direct entre les composants viraux du virion et des HSPG (Barth *et al*., 2003; Vieyres *et al*., 2010). Par ailleurs, il a également été proposé que la région HVR1 de E2 contribue à cette interaction (Koutsoudakis *et al*., 2013; Barth *et al*., 2003; Barth *et al*., 2006). D'autre part, l'apoE, qui est associée aux particules matures du VHC, est également capable d'interagir avec les HSPG, et il a été récemment rapporté que cette apolipoprotéine pourrait être responsable de la liaison des virions VHC aux HSPG (Jiang *et al*., 2012; Jiang *et al*., 2013). Au cours de

cette thèse, ***nous avons donc étudié la contribution relative des protéines d'enveloppe du VHC et de l'apolipoprotéine E dans l'étape initiale d'entrée cellulaire du VHC, en particulier dans l'interaction entre la particule virale et les HSPG. Nous avons également caractérisé les determinants structuraux des HS nécessaires à l'infection par le VHC.***

Dans ce travail, nous nous sommes d'abord intéressés à la région HVR1 de la glycoprotéine d'enveloppe E2 pour déterminer son rôle éventuel dans l'interaction du virion avec les HS. Le haut niveau de variabilité de cette région est en accord avec son accessibilité à la surface de la particule virale, suggérant une interaction potentielle de cette région avec les HSPG comme proposé précédemment sur base d'études effectuées avec des protéines E2 recombinantes (Barth *et al*., 2003). Nous avons donc **ré-évalué** ***le rôle de HVR1 dans l'interaction entre la particule du VHC et les HSPG***. À cette fin, nous avons d'abord construit un virus délété de la région HVR1 dans le contexte de la souche JFH1 (JFH1-ΔHVR1). Pour étudier les propriétés d'interaction du virus JFH1-ΔHVR1 avec les HSPG, nous avons analysé la liaison des virions purifiés à la surface des cellules Huh-7. Nous avons observé que le virus JFH1-ΔHVR1 avait une capacité de liaison similaire au virus de type sauvage (JFH1), ce qui indique que HVR1 n'est pas essentiel pour la liaison initiale des particules de VHC aux cellules d'hépatome. Ensuite, nous avons analysé la capacité de l'héparine, un homologue structural des HS, d'inhiber la fixation du virus VHC à la surface de cellules Huh-7 dans un test de compétition. Nous avons ainsi montré que le virus JFH1-ΔHVR1 était inhibé par l'héparine de manière dose dépendante, indiquant que ce virus conserve ses capacités d'interaction avec les HS présents à la surface de la cellule hôte.

Les expériences précédentes ont été réalisées à l'aide de virus entier et nous avons également voulu déterminer ***si E2-ΔHVR1 dissociée de la particule virale est capable d'interagir avec les HS***. Pour cela, nous avons réalisé des expériences de précipitation avec des billes d'héparine-agarose pré-incubées avec des lysats de cellules infectées par du virus JFH1 ou JFH1-ΔHVR1. Nos résultats ont montré que les glycoprotéines d'enveloppe du VHC sont reconnues de la même manière, que la région HVR1 soit présente ou non, ce qui indique que HVR1 n'est pas nécessaire pour la liaison du complexe E1E2 à l'héparine dans le contexte des protéines virales intracellulaires. Cependant, une réorganisation significative des glycoprotéines d'enveloppe du VHC se produit au cours du bourgeonnement viral et de la sécrétion de la particule (Vieyres *et al*., 2010), nous avons donc également procédé à des expériences de précipitation à l'aide de billes d'héparine-agarose en utilisant cette fois des

lysats de virus JFH1 ou JFH1-ΔHVR1 purifiés. De nouveau, nos résultats ont montré que les glycoprotéines d'enveloppe du VHC sont reconnues de la même manière, que la région HVR1 soit présente ou non dans le contexte des protéines isolées de virus purifié, indiquant que HVR1 n'est pas nécessaire non plus pour la liaison du complexe E1E2 à l'héparine dans le contexte des protéines virales provenant du virion. L'ensemble de ces données indique que la région HVR1 de E2 n'est pas le déterminant principal pour l'attachement à la surface de la cellule et la liaison aux HSPG, cependant nous ne pouvions pas exclure que d'autres régions des glycoprotéines virales peuvent contribuer à la liaison de HS dans le contexte de protéines isolées.

En raison de la présence dans l'enveloppe des glycoprotéines ainsi que des apolipoprotéines à la surface des particules virales du VHC, des résultats contradictoires ont été obtenus en ce qui concerne le facteur déterminant du virion impliqué dans l'étape initiale de la liaison à des cellules hôtes (Barth *et al.*, 2003; Jiang *et al.*, 2012; Jiang *et al.*, 2013). En effet, la glycoprotéine d'enveloppe E2 du VHC et apoE ont été proposées pour être le composant du virion impliqué dans la liaison aux cellules cibles. Par conséquent, pour caractériser notre mutant JFH1-ΔHVR1, nous avons déterminé sa sensibilité à la neutralisation par des anticorps anti-apoE. Nous avons constaté que les deux virus JFH1 et JFH1-ΔHVR1 étaient inhibés par nos anticorps anti-apoE. En raison de l'implication de plusieurs facteurs cellulaires d'entrée dans l'entrée du VHC, des études cinétiques d'inhibition par des anticorps spécifiques ont été utilisées pour déterminer l'implication séquentielle de ces différents facteurs d'entrée (Koutsoudakis *et al.*, 2006; Haberstroh *et al.*, 2008). Ceci peut facilement être réalisé par incubation du virus, en présence d'anticorps ou d'inhibiteurs spécifiques ajoutés à différents temps, avec les cellules hôtes à 4°C, suivie d'un passage à la température de 37°C et d'une mesure de l'infection. Nous avons donc procédé à ce type d'expérience pour déterminer ***si apoE est impliquée dans une étape précoce d'entrée cellulaire du VHC***. En parallèle, nous avons utilisé de l'héparine, un inhibiteur connu de l'étape initiale d'attachement cellulaire du virion. L'effet inhibiteur maximum des anticorps anti-apoE a été observé quand il a été ajouté conjointement avec le virus à 4 °C. En revanche, aucune inhibition n'a été observée quand le virus a été incubé avec les cellules hôtes à 4 °C avant traitement avec l'anticorps anti-apoE. Ces résultats sont similaires aux effets inhibiteurs observés avec de l'héparine, ce qui suggère que l'anticorps anti-apoE inhibe l'attachement des virions à la surface de la cellule. Pour confirmer cette observation, nous avons effectué une mesure directe par qRT-PCR de la liaison des particules virales aux cellules cibles en présence d'anticorps anti-apoE ou

d’héparine. Nous avons ainsi montré que l’anticorps anti-apoE inhibe la liaison du virion aux cellules hôtes. L’ensemble de ces données indique que l'apoE présente à la surface de la particule virale est impliquée dans l'attachement des virions aux cellules hôtes, et ce probablement par interaction avec les HSPG.

Bien que nos données montrent qu’HVR1 n’intervient pas dans l'interaction des particules virales VHC avec les HSPG, nous ne pouvons pas exclure définitivement que d'autres régions de ces glycoprotéines exposées à la surface du virion soient impliquées dans la liaison aux HSPG. En outre, bien que nos données indiquent que l'apoE est impliquée dans la liaison du VHC aux cellules hôtes, il était important de confirmer que l’apoE associée au virion interagit directement avec les HS. Par conséquent, afin de clarifier ***la contribution relative de l'apoE et des glycoprotéines d'enveloppe dans l'interaction du VHC avec les HS***, nous avons réalisé des expériences de précipitation avec des billes d'héparine-agarose incubées en présence de particules virales purifiées pré-incubées avec des compétiteurs qui ciblent l'interaction apoE-HS ou en présence de différents anticorps monoclonaux neutralisants anti-VHC. Nous avons constaté que les anticorps neutralisants ciblant le VHC n’empêchent pas la liaison des virions à l'héparine. En revanche, la pré-incubation des virions avec des anticorps anti-apoE inhibe fortement la liaison des virions aux billes d'héparine-agarose. En outre, l'incubation avec un peptide dérivé de apoE montre que celui-ci permet la liaison apoE-HS et inhibe ainsi l'interaction des virions du VHC avec les billes d'héparine-agarose. L’ensemble de ces résultats est en faveur d'un rôle majeur de l'apoE dans les interactions entre la particule virale et les HS.

Dans une seconde partie de ce travail, nous avons également voulu caractériser ***les déterminants structuraux des HS impliqués dans l’interaction avec les virions VHC***. Nous avons d'abord déterminé la longueur minimale des HS, nécessaire pour inhiber l'infection virale en utilisant des oligosaccharides d'une longueur définie dérivés de l'héparine. Nous avons constaté que la pré-incubation du virus avec des oligosaccharides de dp10 ou dp12 inhibe l'infection par le VHC à des niveaux similaires à l'héparine, ce qui suggère qu'un minimum de 10 saccharides est nécessaire pour l'interaction du VHC avec les HS.

Pour mieux caractériser les déterminants des HS impliqués dans la liaison du VHC, nous avons identifié les HS sulfotransférases exprimées dans les cellules Huh-7, cellules cibles du VHC. La biosynthèse des HS est divisée en trois grandes étapes: l'initiation de la chaîne, la

polymérisation et la modification. L'étape d'initiation est caractérisée par le lien entre un tétrasaccharide et la partie protéique du protéoglycane. Ensuite, le squelette d'HS est formé par l'assemblage alternatif des résidus glucuronate (GlcUA) et N-acétylglucosamine (GlcNAc). Lorsque la chaîne est assemblée, elle subit une série de modifications catalysées par une C5 épimérase et plusieurs sulfotransférases. La première modification de la chaîne se produisant est l'élimination du groupe N-acétyl présents sur des GlcNAc et l'addition d'un groupe N-sulfo. Cette réaction est orchestrée par des membres de la famille des *N*-deacetylases-*N*-sulfotransferases (NDST). D'autres modifications des HS comprennent une épimérisation de certains glucuronates en résidus d'iduronate, puis une addition de groupements sulfates en C2 de l'acide uronique par la 2-*O*-sulfotransferase (2-OST), et en C6 et/ou en C3 des GlcN par les enzymes 6-OST et 3-OST, respectivement. Il n'existe qu'une seule isoforme de la 2-OST. Par contre, il existe trois isoformes pour la 6-OST et sept pour la 3-OST. Nous avons donc analysé le profil d'expression des isoenzymes qui interviennent dans la sulfatation de HS. Nous avons détecté des niveaux élevés d'ARNm codant pour NDST1 dans des cellules Huh-7. L'expression de NDST2 était par contre très faible, tandis qu'aucun transcrit n'a été dédecté pour les enzymes NDST3 et NDST4. Nous avons également constaté un niveau élevé d'expression des transcrits de la 2-OST ainsi qu'une expression modeste des transcrit 6-OST1 et 6-OST2, deux isoenzymes impliqués dans la même réaction de 6-O-sulfatation. Finalement, nous avons également détecté un faible niveau de 3-OST3A, 3-OST3B et 3-OST5 dans des cellules Huh-7.

Pour déterminer ***si des motifs particuliers de sulfatation sont importants pour les interactions entre le virus VHC et les HS***, nous avons effectué une infection en présence d'héparines chimiquement modifiées. Nous avons ainsi montré que l'héparine 2-O-désulfatée est toujours capable d'inhiber l'infection par le VHC à un niveau cependant légèrement inférieur à l'inhibition induite par l'héparine non modifiée. En revanche, les héparines N-désulfatée et 6-O-désulfatée utilisées aux mêmes concentrations étaient incapables d'inhiber l'infection par le VHC. Ces résultats suggèrent que les groupes N- et 6-O-sulfo sont importants pour l'interaction avec le VHC. Pour confirmer ces résultats, nous avons modifié, par interférence à l'ARN, l'expression des enzymes impliquées dans la formation des HS. Nous avons ainsi ciblé les enzymes qui étaient exprimées en cellules Huh-7. Ainsi, nous avons utilisé des ARN interférents ciblant la NDST1, la 2-OST et une combinaison d'ARN interférents ciblant les deux isoformes de la 6-OST (6-OST1 and 6-OST2). Ensuite, nous avons analysé l'effet de la réduction de l'expression de ces enzymes sur l'infection par le

VHC. Nous avons observé une diminution de 60% de l'infection par le VHC dans des cellules traitées par des ARN interférents ciblant NDST1, ce qui indique que la N-sulfatation est nécessaire pour l'interaction entre le VHC et les HS. L'infection par le VHC n'a par contre pas été affectée par la réduction de l'expression de la 2-OST, tandis que l'inactivation des deux isoformes de 6-OST réduisait l'infection par le VHC. Ces observations sont en accord avec les résultats obtenus avec les héparines désulfatées et soutiennent l'idée que la N- et 6-O-sulfatation, mais pas 2-O-sulfatation, sont nécessaires pour l'infection par le VHC.

En conclusion, nous avons étudié la contribution relative des protéines d'enveloppe du VHC et apoE dans l'étape initiale de l'entrée cellulaire du VHC, en particulier au niveau de l'étape de liaison aux HSPG, et nous avons caractérisé les déterminants structuraux des HS nécessaires pour l'infection par le VHC. Nos résultats soutiennent l'hypothèse que l'apoE associée au virus est responsable de l'interaction du VHC avec les HS. Nous avons également constaté que la N- et 6-O-sulfatation des HS, mais pas 2-O-sulfatation, sont nécessaires pour l'infection par le VHC. Enfin, nous avons également montré que la taille minimale des oligosaccharides d'HS requise pour l'infection par le VHC est un decasaccharide. En conclusion, l'ensemble de ces données indique que le VHC détourne l'apolipoprotéine E pour initier son interaction avec des structures d'HS spécifiques.

Le mécanisme d'entrée du virus de l'hépatite C est complexe impliquant de nombreux partenaires aussi bien viraux que cellulaires. Nous avons pu élucider, en partie, une des premières étapes de l'interaction du virus avec sa cellule hôte. Une meilleure compréhension de ces mécanismes pourra permettre d'envisager de nouvelles cibles thérapeutiques.

Mots-clés: Virus de l'Hépatite C, le Sulfate d'Héparane, l'entrée du virus, l'apolipoprotéine E

ABSTRACT

Hepatitis C virus (HCV) entry involves binding to cell surface heparan sulfate (HS) structures. However, due to the lipoprotein-like structure of HCV, the exact contribution of virion components to this interaction remains controversial. Here, we investigated the relative contribution of HCV envelope proteins and apolipoprotein E in the HS-binding step. Deletion of hypervariable region 1, a region previously proposed to be involved in HS-binding, did not alter HCV virion binding to HS, indicating that this region is not involved in this interaction. Neutralizing monoclonal antibodies recognizing different regions of HCV envelope glycoproteins were also used in a pull-down assay with beads coated with heparin, a close HS structural homologue. Although isolated HCV envelope glycoproteins could interact with heparin, none of these antibodies was able to interfere with virion-heparin interaction, strongly suggesting that, at the virion surface HCV envelope glycoproteins are not accessible for HS binding. In contrast, results from kinetic studies, heparin pull-down and inhibition experiments with anti-apolipoprotein E antibodies indicate that this apolipoprotein plays a major role in HCV-HS interaction. Finally, characterization of HS structural determinants required for HCV infection by silencing enzymes involved in the HS biosynthesis pathway and by competition with modified heparin indicated that N- and 6-O-sulfation but not 2-O-sulfation are required for HCV infection, and that the minimum HS oligosaccharide length required for HCV infection is a decasaccharide. Together, these data indicate that HCV hijacks apolipoprotein E to initiate its interaction with specific HS structure.

Key words: Hepatitis C virus, Heparan sulfates, viral entry, apolipoprotein E

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ABBREVIATIONS

AAV-2	Adeno-associated virus-2
AcLDL	Acylated LDL AcLDL
ALT	Alanine aminotransferase
APs	Amphipathic DNA polymers
BBB	Blood-brain barrier
BVDV	Bovine viral diarrhoea virus
CAR	Coxsackie-adenovirus receptor
CBAs	Carbohydrate-binding agents
CD81	Cluster of differentitation 81
CLDN1	Claudin1
CMFDA	5-chloromethylfluorescein diacetate
CM	Chylomicron
CMV	Cytomegalovirus
CNS	Central nerve system
CSF	Cerebrospinal fluid
CS	Chondroitin sulfate
CVB3	Coxsackie virus type B3
CyPB	Cyclophilin B
DAAs	Direct-acting antivirals
DAPI	4’,6-diamidino-2-phenylindole
DC-SIGN	Dendritic cell specific ICAM-3 grabbing non-integrin
DGAT1	Diacylglycerol Acyltransferase 1
DRMs	Detergent-resistant membranes
DS	Dermatan sulfate
EAR	Early airway responsiveness
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EL	Extracelluar loops
EL	Endothelial lipase
EMCV	Encephalomyocarditisvirus
ER	Endoplasmic reticulum
EphA2	Ephrin receptor A2
FASN	Fatty acid synthase
Fah	Fumarylacetoacetate hydrolase
FGF-2	Fibroblast growth factor-2
FMDV	Foot and Mouse Disease Virus
FRET	Fluorescence resonance energy transfer
GalNAc	N-acetyl-glucosamine
GlcA	D-glycuronic acid
GT1	Genotype 1
GPI	Glycosylphosphatidylinositol
GFP	Green flurorescent protein
GlcA	Glucuronic acid
GlcNAc	N-acetyl-D-glucosamine
GPIHBP1	Glycosyl phosphatidyl inositol-anchored high-density lipoproteinbinding protein 1
HB-GAM	Heparin binding growth-associated molecle
HCC	Hepatocellular carcinoma
HCVpp	HCV pseudoparticles
HDL	High density lipoprotein

HFLCs	Human fetal liver cells
HHV	Human herpevirus
HIV	Human immunodeficiency virus
HIVD	HIV dementia
HL	Hepatic lipases
HLP	Type III hyperlipoproteinemia
HPV	Human papillomavirus
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycans
HTGL	Hepatic triglyceride lipase
HTLV-I	Human T cell Leukemia Virus type I
HVEM	Herpesvirus entry mediator
HVR1	Hypervirable region 1
IdoA	L-iduronic acid
IEG	Immediate early gene
IFN-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
IRES	Internal ribosomal entry site
ITPA	Inosine triphosphatase
JEV	Japanese encephalitis virus
KS	Keratan sulfate
LAR	Late airway responsiveness
LCAT	Lecithin: cholesterol acyltransferase
LDLr	Lipoprotein receptor
LDs	Lipid drops
LDLR	Lipoprotein receptor
LEL	Large extracellular loop
LMWH	Low molecular weight fractions of heparin
LPL	Lipoprotein lipase
Luc	Luciferase
LVPs	Lipo-viro-particles
MAVS	Mitochondrial antiviral signaling
MAFs	Membrane-associated foci
MMTV	Mouse mammary tumor virus
MLV	Murine leukermia virus
MTP	Microsomal triglyceride transfer protein
NDSTs	N-deacetlyase/N-sulfotransferases
NK	Natural killer
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NPC1L1	Niemann-Pick C1-like1
NTRs	Non-translated regions
OCLN	Occludin
ORF	Open reading frame
oxLDL	Oxidized LDL
PAPS	3'-phosphoadenyl-5'-phosphosulfate
PBMCs	Peripheral blood mononucleated cells
PEG-IFNα	Pegylated interferon alfa
PHH	Primary human hepatocytes
PLE	Protein-losing enteropathy
PPARα	Peroxisome proliferator-activated receptor α

RBV	Ribavirin
RCs	Replication complexes
RCT	Reverse cholesterol transport
RSV	Respiratory syncytial virus
RTKs	Receptor tyrosine kinases
SEL	Small extracellular loop
SL	Stem-loop
SM	Sphinogomyelin
SNPs	Single nucleotide polymorphisms
SPR	Surface plasmon resonance
SVR	Sustained virological response
TEMs	Tetraspanin-enriched microdomains
TfR1	Transferrin receptor 1
TG	Triglyceride
TJ	Tight junction
TRL	Triglyceride-rich lipoprotein
TMD	Transmembrane domain
uPA/SCID	Homozygous urokinase-type plasminogen activator/severe combined immuodeficiency
VSV	Vesicular stomatitis virus
VEGF	Vascular endothelial growth factor
VV	Vaccinia virus
WNV	Western nile virus
Xyl	Xylose
2OST	2-O-sulfo-transferase
3OST	3-O-sulfo-transferase
6OST	6-O-sulfo-transferase

INTRODUCTION

I. General introduction of Viral Hepatitis C

Hepatitis is the general term which refers to inflammation of the liver. There are many causes of hepatitis, which can either be infectious or non-infectious. Viral hepatitis C is caused by hepatitis C virus (HCV) infection, which was first called non-A, non-B post-transfusion hepatitis (Alter *et al*., 1982). However, due to difficulties in propagating this virus in cell culture, it took more than a decade to identify the infectious agent responsible for this newly-emerged viral hepatitis. This was achieved by molecular cloning, and the virus was named HCV (Choo *et al*., 1989).

1. Hepatitis C epidemiology

HCV infection is still a global public health problem, which causes severe liver diseases including fibrosis, cirrhosis, liver failure and hepatocellular carcinoma (HCC) (Saito *et al*., 1990). Up to 3% of the world population, around 170 million people are currently infected, and more than 350,000 people die of HCV-related diseases each year (Mohd *et al*., 2013). Hepatitis C, together with hepatitis B, accounts for 75% of all cases of liver diseases worldwide (Ananthakrishnan *et al*., 2006).

Molecular epidemiological studies show that HCV prevalence varies from one region to another in the world. Middle east and North Africa have high levels of prevalence (>2.9%); Central and Southern Asia, and sub-Saharan Africa have moderate prevalence (2.0%-2.9%); whereas Europe, Latin America and North America have low levels of prevalence (less than 1%) (Fig.1). The highest prevalence occurs in the eastern Mediterranean region, especially in Egypt, where HCV infection is reported in 10 to 30% of the general population (Cornberg *et al*., 2011).

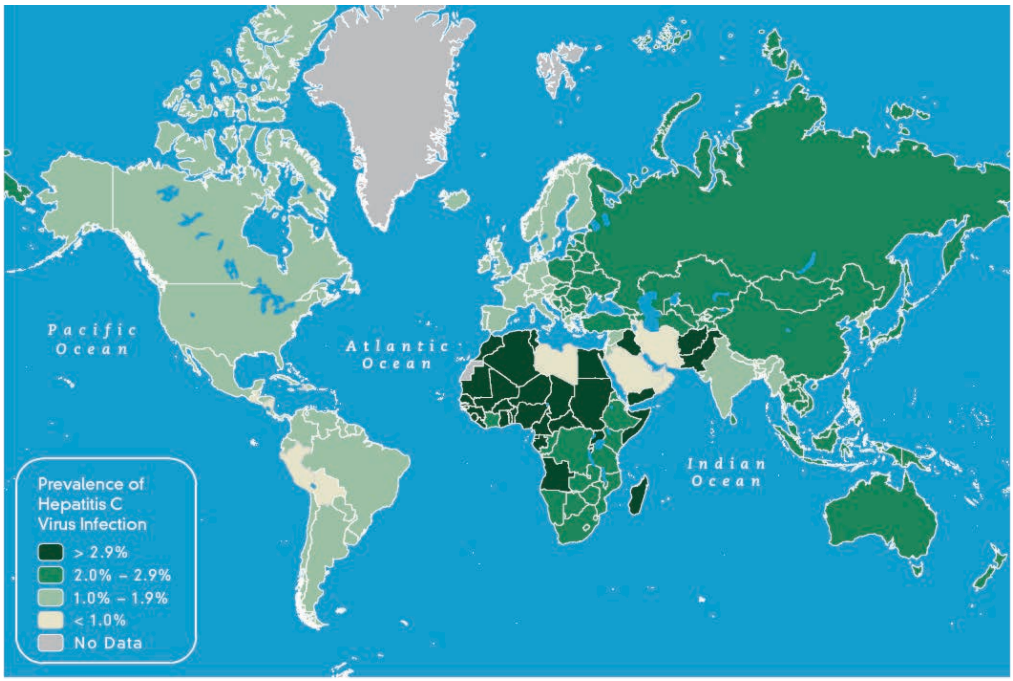


Fig. 1: Prevalence of HCV infection (source: 2012 CDC)

The global prevalence of HCV infection among pregnant women is estimated to be between 1%-8%, and in children it's between 0.05%–5%. The absence of an available vaccine or approved therapy during pregnancy makes the prevention of HCV vertical transmission still impossible. However, HCV has a low rate of vertical transmission (3–5%) and a high rate of spontaneous clearance (25–50%) in children, which is the opposite of what is observed for HBV infections. These observations together with a delayed morbidity have resulted in HCV infection being ignored in pregnant women and their newborn babies (Arshad *et al*., 2011). Of note, factors such as high maternal HCV viral load and co-infection with HIV-1 can increase the mother-to-child transmission (Thomas *et al*., 1998), while HCV genotype, the mode of delivery and breastfeeding are not related with vertical transmission of this virus (Indolfi & Resti, 2009).

HCV is mainly transmitted by exposure to contaminated blood. This may happen through transfusion of HCV-infected blood and blood products, contaminated injections during medical procedures, and sharing injecting needles and syringes among drug users. Another predominant mode of transmission is high-risk sexual contact. Though sexual transmission accounts for a minority of cases on record, in recent years acute hepatitis C has become a considerable problem in HIV-infected homosexual males (Lopez-Dieguez *et al*., 2011).

2. Natural history of HCV infection

Acute HCV infection

The incubation period for HCV infection varies from 6 to 10 weeks, but long incubation periods can occur in the cases that only small amounts of viral loads have been transmitted (Mosley *et al*., 2005). Most patients are asymptomatic during the acute phase, making the diagnosis very challenging. In symptomatic patients, fatigue, nausea, vomiting, abdominal pain, appetite loss, mild fever, itching or myalgia that are not specific, usually appear during the sixth or seventh week and resolve within a few weeks. Jaundice is the only specific, liver related clinical sign in the symptomatic course of an acute HCV infection (Loomba *et al*., 2011). In contrast to acute HBV infection, acute HCV infection is self-limited only in 15-40% of patients, and most infected individuals who cannot achieve spontaneous viral clearance, progress to chronic infection (Micallef *et al*., 2006).

Chronic HCV infection

During acute infection, HCV RNA is usually detected in the serum before anti-HCV antibody. Chronic HCV infection is diagnosed when HCV RNA persists in the blood for more than six months after the onset of acute infection and HCV antibody is positive. As shown in Fig.2, among the 60–85% chronically infected patients, 15–30 % of them progress to cirrhosis and 1–3% will need liver transplantation or die from cirrhosis or liver cancer per year. Generally, a typical disease course from infection to symptomatic liver diseases takes as long as 20-30 years. Many chronic HCV infections do not exhibit symptoms during this period, though some individuals experience fatigue, depression or other extrahepatic manifestations of HCV infection (Galossi *et al*., 2007).

Factors that promote the progression of chronic hepatitis include alcohol use, gender, age at the time of infection, and immune deficiency (Lauer & Walker, 2001). Importantly, co-infection with HIV and/or HBV are significant risk factors for liver fibrosis (Benhamou *et al*., 1999; Raimondo *et al*., 2005; Vermehren *et al*., 2012). HCV viral load and genotype do not seem to affect the progression rate. However, some evidence show that infection with HCV of genotype 3 is associated with steatosis which can accelerate the fibrosis progression (Bochud *et al*., 2009). Obesity and diabetes may also lead to more rapid fibrosis progression, whereas liver fibrosis is believed to be progressive, and largely irreversible by the antifibrotic treatments currently used. Importantly, it is the progression of fibrosis that ultimately leads to

severe architectural damage of the liver and the disruption of metabolic functions of the liver.

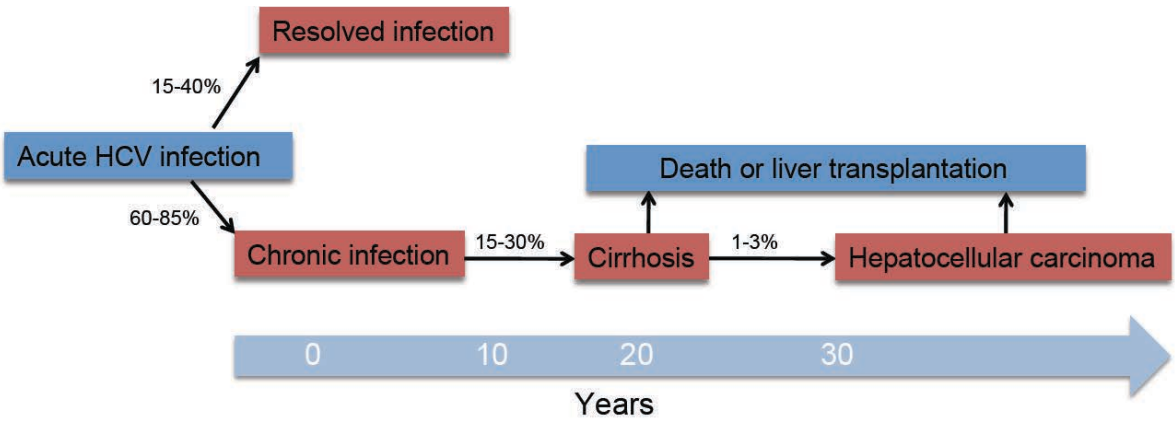


Fig. 2: The natural history of HCV infection.
Adapted from (Vassilopoulos & Calabrese, 2012).

3. Diagnosis and Treatment of HCV infection

Since HCV infection is typically mild in its early phase, it is often not diagnosed, or diagnosed in the context of screening of blood donors or testing as part of the application for various types of insurance. Otherwise HCV infection is often not detected until its chronic stages when it has caused severe liver diseases.

Once an acute hepatitis C is suspected, the presence of both anti-HCV antibodies and HCV RNA should be tested. When HCV RNA is detected in seronegative patients, acute hepatitis C is very likely. HCV RNA becomes detectable in serum within 7 and 21 days after the viral transmission, whereas anti-HCV antibodies can be detected in 90% of the patients 3 months after infection (Farci *et al.*, 1991; Hoofnagle, 1997; Mosley *et al.*, 2005). Viral RNA detection tests can be qualitative or quantitative. Due to their sensitivity, the qualitative tests are used to confirm viremia and to assess the ultimate treatment response. On the other hand, the quantitative tests are applied to determine HCV viral load, to monitor response to treatment, and to determine the presence of infection in immunocompromised patients (Germer & Zein, 2001). Many commercial assays are available for qualitatively detecting or quantitatively measuring HCV RNA level in the blood. The latest quantitive tests are nearly as sensitive as the old qualitative tests, thus there is no more need to use the qualitative tests. This makes HCV RNA testing simple and efficient, settling the matter at one go. The HCV RNA tests

currently available exploit either reverse transcription-polymerase chain reaction (RT-PCR) technology or branched DNA signal amplification. It is important to use the same test serially because the results are not directly comparable from one test to another (Chevaliez & Pawlotsky, 2006; Scott & Gretch, 2007).

HCV genotyping is mandatory in every patient under consideration for antiviral therapy (Bowden & Berzsenyi, 2006). It can be conducted with a commercial assay using PCR products on genotype-specific hybridization probes or through direct sequencing (Stuyver *et al*., 1993; Holland *et al*., 1998; Giannini *et al*.,1999; Scott & Gretch 2007). Other laboratory tests measuring blood liver enzyme concentrations are also often used for monitoring the disease. Notably, the level of serum alanine transaminase (ALT) elevation does not correlate with liver histologic lesions, and may be normal in any stage of chronic hepatitis C (Kamili *et al*., 2012).

For the treatment of HCV infection, the aim is to prevent clinical complications and death from the infection. Treatment of patients with acute genotype 1 (GT1) infection is especially important, because the treatment can be shortened and appear more efficacious, compared to treatment during the chronic phase (Ghany *et al*., 2011; Fox & Jacobson, 2011). The standard therapy of chronic HCV infection is the combination of pegylated interferon alfa (PEG-IFNα) and ribavirin (RBV). Several types of virological responses to the antiviral treatment may occur (Fig.3), the most important is the sustained virological response (SVR), which is defined as undetectable level of HCV RNA at least 24 weeks after the completion of treatment. SVR can be achieved in 40-50% patients infected with GT1, and up to 80% of those infected with genotypes 2 or 3 (Manns *et al*., 2001; Fried *et al*., 2002; Zeuzem *et al*., 2009). Recently, an HCV protease inhibitor (either telaprevir or boceprevir) was added to the standard therapy for treatment of HCV GT1 infection (Zeuzem *et al*., 2011). The addition of these direct-acting antivirals (DAAs) increase the SVR to 70-80% in GT1 infected patients, however, a significant number of patients are not eligible for this triple therapy in a real clinical setting, particularly patients with severe liver disease (Maasoumy *et al*., 2013). During the treatment, the kinetics of the HCV RNA decline is a strong predictor of treatment response. Monitoring HCV RNA level in the blood at week point 4, 12 and 24 is important for a response-guided treatment approach not only for PEG-IFN/RBV but also for the new triple therapy (Ghany *et al*., 2011).

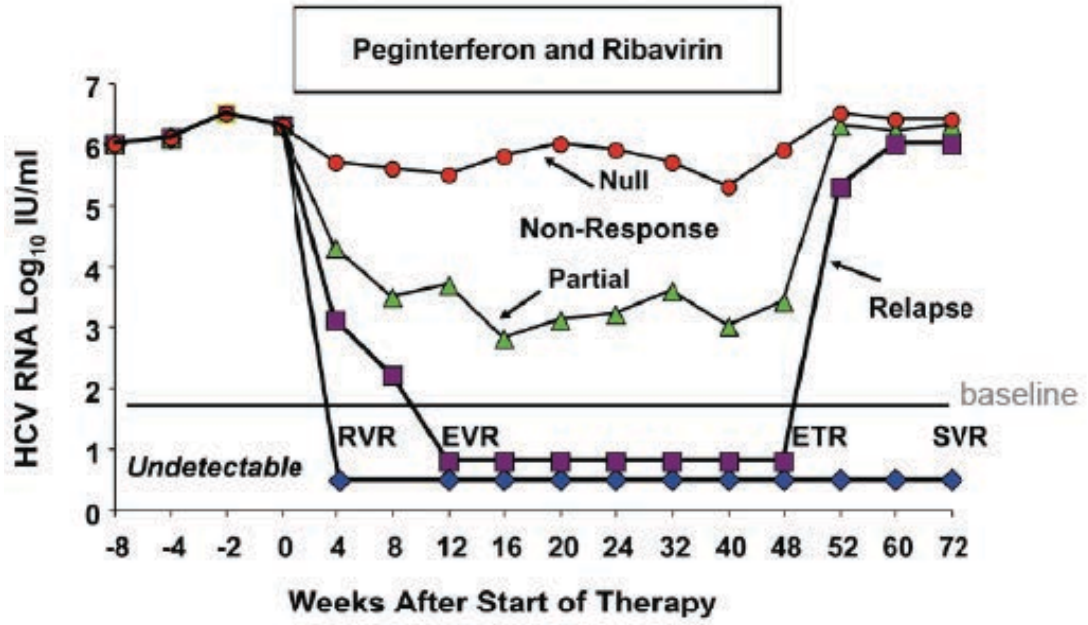


Fig. 3: Graphic display of virological responses

RVR, rapid virological response (undetectable HCV RNA at week 4 of treatment using a sensitive PCR based assay); **EVR**, early virological response (more than 2 log reduction in HCV RNA level compared to baseline HCV RNA level or HCV RNA negative at treatment week 12); **SVR**, sustained virological response (HCV RNA negative 24 weeks after cessation of treatment); **Relapse**, reappearance of HCV RNA in serum after therapy is discontinued; **Non-Responder**, failure to clear HCV RNA from serum after 24 weeks of therapy; Partial nonresponder, 2 log decrease in HCV RNA but still HCV RNA positive at week 24; **Null**, failure to decrease HCV RNA by 2 logs after 24 week of therapy (Ghany *et al*., 2009; Ghany *et al*., 2011).

Viral load is an independent determinant of treatment response. The lower HCV RNA level is in the blood (viral load), the more likely the virus can be eradicated from the bloodstream. However, many factors can affect the ultimate outcome of antiviral treatment. Viral factors including HCV genotype, HCV 5' NTR (non-translated region), and genetic variations exert significant influence on the treatment response (Imran *et al*., 2013). Studies show that patients infected with HCV subgenotype 1a have a more favorable response than patients with sub-genotype 1b infection (Pellicelli *et al*, 2012). By comparing HCV genomic sequences between patients who achieved SVR and those who do not, many specific regions in HCV core, p7, NS2, NS3 and NS5A genes were identified to be strongly associated with the outcome of therapy (Wohnsland *et al*., 2007; EI-Shamy, *et al*., 2008). The most important

region is the interferon sensitivity determining region (ISDR), which is located in HCV NS5A (Akuta *et al*., 2010; Kadokura *et al*., 2011). The outcome of treatment not only depends on viral factors, but also depends on host factors. Host genetic factors such as Interleukin-28B (IL-28) and inosine triphosphatase (ITPA) polymorphism have been found to be associated with disease progression, as well as treatment response and adverse effects (Clark & Thompson, 2010; Miyamura *et al*., 2012). Single nucleotide polymorphisms (SNPs) of host genes, especially within chromosome 19, near the IL28A, IL28B and IL29 genes are significantly linked to the SVR (Tanaka *et al*., 2009; Tillmann *et al*., 2010). People with a particular sequence in IL28B gene, called CC genotype, are more likely (50-80%) to be cured from HCV infection (Ge *et al*., 2009; Thomas *et al*., 2009). Other host factors including age, race, sex, obesity, alcohol abuse, insulin resistance, hepatitc steatosis are also predictors for the interferon based antiviral therapy (Imran *et al*., 2013).

Though the addition of DAAs significantly increases the SVR in HCV GT1 infection, the treatment is successful only in 70-80% of treated individuals (Bacon *et al*., 2011; Poordad *et al*., 2011). Moreover, both IFN and RBV have potential side effects. Flu-like symptoms including fatigue, headaches, fever, muscular and joint aches are very common. Patients may also experience stomach symptoms such as nausea, decreased appetite, or mental symptoms like depression, insomnia, irritability and concentration difficulties. Those side effects such as anaemia, neutropenia and thrombocytopenia may require dose reduction or cessation of treatment (Zeuzem *et al*., 2011; Lok *et al*., 2012). Recently, there have been major advances in hepatitis C treatments with the licensing of the first DAAs and large numbers of ongoing clinical trials with various DAAs showing high potency, favorable tolerability profile, higher barrier to resistance, shortened treatment duration and all oral regimen (Pawlotsky, 2014). The arrival of these new antiviral molecules is therefore progressively changing the landscape of hepatitis C treatment.

4. Molecular biology of HCV

4.1 HCV classification and genotype distribution

HCV is a small enveloped, postive-sense RNA virus. This virus belongs to the Hepacivirus genus in the *Flaviviridae* family, which includes the classical flaviviruses such as yellow fever virus, dengue virus and tick-borne encephalitis virus, the animal pestiviruses like bovine viral diarrhoea virus and GB viruses, including GBV-A, GBV-B, GBV-C and GBV-D (Simmonds, 2013).

Based on the phylogenetic analyses of the core/E1 and NS5 region of viral genome, HCV is classified into 7 major genotypes, numbered from genotype 1 to genotype 7 (Simmonds *et al*., 1993; Simmonds *et al*., 2005; Murphy *et al*., 2007). The variance of nucleotide sequence between different genotypes is about 30%. Within each genotype, the subtypes differ from each other by 20-25% at the nucleotide level. HCV genotypes and subtypes spread very differently in different regions in the world. For example, genotype 1, 2, and 3 are widely distributed in the USA, Europe, Australia and East Asia, while genotype 4 is very common in Middle East, Egypt, and Central Africa. Genotype 5 is prevalent in South Africa, and genotype 6 predominates in Southeast Asia (Simmonds *et al*., 2004). Recently, genotype 7 was found in central Africa (Murphy *et al*., 2007).

Fig. 4: HCV genotypes and subtypes geographic distribution.
Source: (Nguyen & Keeffe, 2005)

HCV genotype information is considerably important, because it offers critical reference in HCV medical treatment. For example, the treatment duration, dose of ribavirin and whether an HCV protease inhibitor will be added to the standard therapy are affected by the genotype (Slavenburg *et al*., 2009; Kwo, 2011; Mangia & Mottola, 2012). Clinically, HCV genotypes 1 and 4 are more resistant to standard of care treatment and are more difficult to be cleared from patients. Regarding HCV genotype and disease progression, it has been suggested that certain genotypes may lead to a more severe disease progression. Clinical trials show that patients infected with genotype 3a are more prone to develop liver steatosis (Mirandola *et al*., 2009).

For HCV subtypes, the difference of their roles in the disease progression is not clear, but differences in the outcome of treatment with new HCV protease inhibitor combination therapy (boceprevir and telaprevir) are visible. Recent studies have shown that both these protease inhibitors function better in patients infected with genotype 1b than those infected with genotype 1a (Jacobson *et al*., 2011; Zeuzem *et al*., 2011; Pearlman & Traub, 2012).

4.2 HCV genome and viral proteins

HCV genome

HCV genome is a 9.6-kb uncapped linear positive sense single-stranded RNA (ssRNA) molecule, consisting of 5', 3' non-translated regions (NTRs) and a large open reading frame (ORF), which encodes a precursor polyprotein of ~3000 amino acids (Choo *et al*., 1991). This polyprotein is co- and post- translationally cleaved into at least 10 viral proteins including 3 structural proteins (core and envelope proteins E1 and E2) and 7 non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Fig.5) (Moradpour *et al*., 2007; Bartenschlager *et al*., 2013). In addition, an alternative ORF has been identified, which overlaps the core gene in the +1 frame, resulting in the production of ARFP/Core+1/F protein (Xu *et al*., 2001; Vassilaki & Mavromara, 2003). HCV structural proteins are components of the virion and required for HCV infection, while the nonstructural proteins are essential for viral replication as well as in virion assembly (Moradpour *et al*., 2007; Bartenschlager *et al*., 2013).

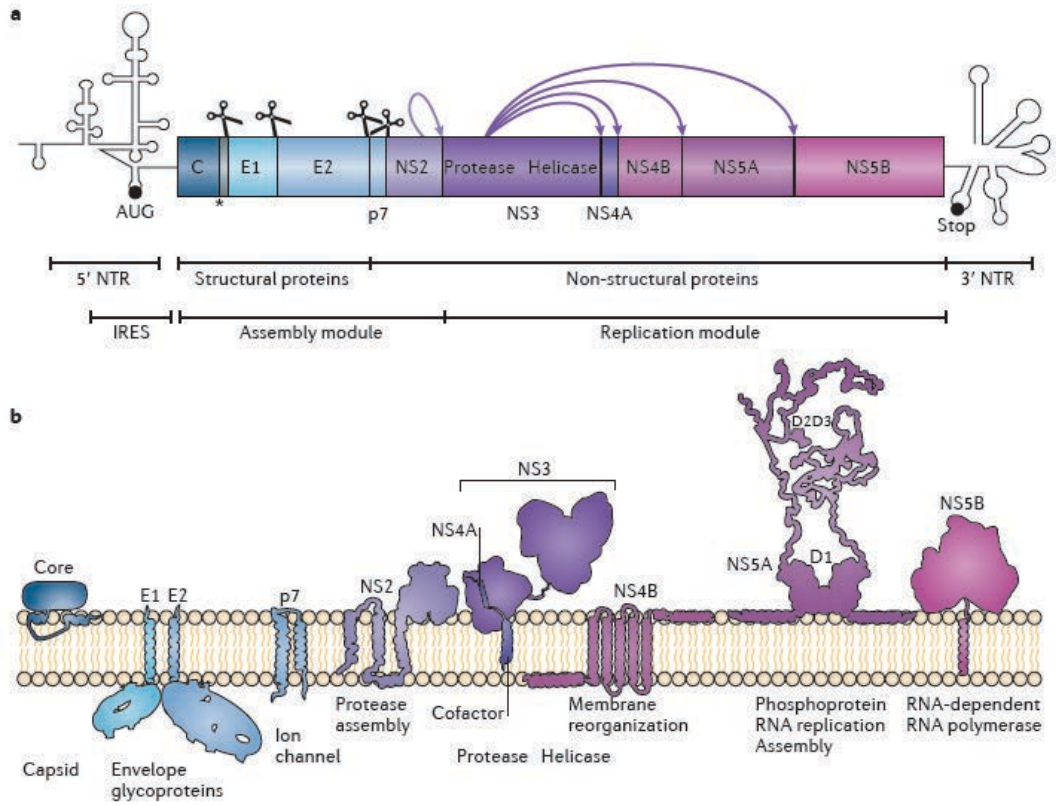


Fig. 5: HCV genome and the viral proteins and their protein functions.

a. HCV genome and encoded proteins **b**. Membrane topology of viral proteins and their founction (Bartenschlager *et al*., 2013).

HCV 5'NTR is the most conserved region of HCV genome. It is located upstream of the ORF, containing 341-344 nucleotides(nt) which forms 4 highly secondary structured domains, named domain I to IV. Domains II, III and IV (Fig.5), together with the first 12 to 30 nt of the core protein coding region, constitute the internal ribosomal entry site (IRES). HCV IRES has the capacity to form a stable pre-initiation complex by directly binding the 40S ribosomal subunit without the need of canonical translation initiation factors, and mediates the cap-independent translation of the HCV ORF (Honda *et al*., 1996). The entire 5'NTR is required for HCV RNA replication, and the crucial elements are located in domains I and II (Friebe *et al*., 2001; Kim *et al*., 2002).

HCV 3'NTR contains approximately 225nts. It is organized in three regions, including a variable region of approximately 40nts, a long poly (U/UC) tract, and a highly conserved 98nt 3'-terminal domain (3' X tail). Part of the poly (U/UC) tract and the 3' X region, by interacting with a stem-loop (SL) region of the RNA element in NS5B were found to be essential for

RNA replication. The remaining sequence of the 3'NTR seems to strengthen viral replication (Yi & Lemon, 2003; Murayama *et al*., 2010).

Viral proteins

Core is a structural protein that takes part in the formation of the capsid shell of HCV virion. It is derived from the N-terminus of the polyprotein (Fig.5), released as a precursor protein of 23kDa, and the final processing product is 21kDa. The mature p21 core protein consists of two domains. The N-terminal domain (D1) binds to the genomic RNA and initates the caspid assembly, whereas the C-terminal domain (D2) targets core to the cytosolic lipid droplets (LDs) in infected liver cells (McLauchlan *et al*., 2002; McLauchlan, 2009). Importantly, the association of core with LDs is crucial for the virus production (Boulant *et al*., 2007; Lyn *et al*., 2013). The localization of core on the surface of LDs also seems to contribute to the core-induced steatosis, which is characterized by the accumulation of intracellular LDs (Moriya *et al*., 1997). As noted earlier, liver steatosis caused by HCV infection occurs very frequently in genotype 3 infected patients (Kumar *et al*., 2002; Poynard *et al*., 2003; Patton *et al*., 2004), and a recent study has shown that *in vitro* expression of genotype 3 core, but not genotype 1b, leads to the formation of large LDs in hepatocytes (Clement *et al*., 2011).

HCV **envelope glycoproteins E1 and E2** are the essential components of viral envelope. Being exposed on the virion, envelope proteins play major roles in HCV entry by interacting with its receptors or co-receptors on the cell surface of hepatocytes. E1 and E2 are cleaved from the polyprotein by an ER signal peptidase, and both are type I transmembrane proteins, containing a large N-terminal extracellular domain and a small C-terminal hydrophobic transmembrane domain (TMD). The ectodomains of E1 and E2 are highly glycosylated, with up to 5 and 11 N-glycosylation sites, respectively (Fig.6) (Lavie *et al*., 2007). These N-linked glycans are involved in the folding of envelope glycoproteins, by association with ER chaperones (Dubuisson & Rice, 1996; Choukhi *et al*., 1998). Several glycans on E2 were recently shown to alter the recognition of neutralizing epitopes, reducing the sensitivity of HCV to antibody neutralization (Helle *et al*., 2010). This type of immune evasion aided by the glycan shielding of viral envelope glycoproteins is also observed for other viruses, and it leads to the idea of targeting these glycans for the development of novel antiviral treatments (Aguilar *et al*., 2006, Wei *et al*., 2010; McLellan *et al*., 2011). The transmembrane domains of E1 and E2 contain ER retention signals (Cocquerel *et al*., 1998; Cocquerel *et al*., 1999). The

subcellular accumulation of HCV envelope proteins in the ER compartment has been shown to be indispensable for the formation of E1E2 heterodimers and the assembly of envelope proteins (Cocquerel *et al*., 2000; Op De Beeck *et al*., 2004). Interestingly, it has been shown that intracellular forms of E1 and E2 assemble as non-covalent heterodimers, whereas the mature secreted E1 and E2, which are incorporated into infectious virions, form large covalent complexes stabilized by disulfide bridges (Dubuisson *et al*., 2000; Krey *et al*., 2010; Vieyres *et al*., 2010).

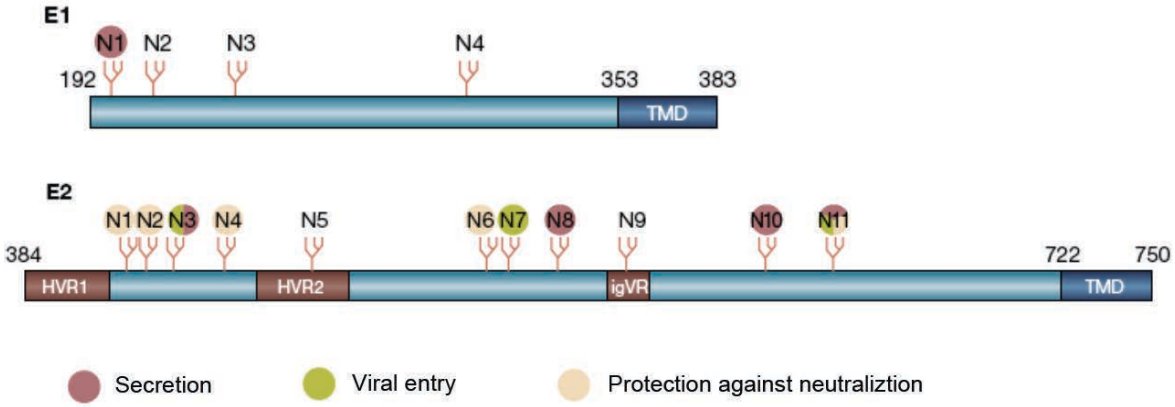


Fig. 6: Diagrammatic sketch of HCV envelope glycoproteins E1and E2 (Helle *et al*., 2009)

P7 is a 63-residue membrane-spanning protein, localized in the ER, with its N- and C-termini located in the ER lumen, and a short hydrophilic loop revealed in the cytosol (Carrère-Kremer *et al*., 2002). Both N- and C- termini of p7 are cleaved from the polyprotein by an ER signal peptidase of the host cell. P7 belongs to a protein family called viroporins (Nieva *et al*., 2012) and it oligomerizes to form ion channels with cation selectivity (Premkumar *et al*., 2004). P7 is not predicted to be required for RNA replication, since HCV sub-genomic replicons with minimum genome requirement from NS3 to NS5B can support efficient RNA replication (Bartenschlager & Lohmann, 2000). However, p7 was found to play an important role in virus infection in cell culture and in chimpanzees (Sakai *et al*., 2003; Jones *et al*., 2007). It has also been shown to be necessary for virus assembly and release in a genotype specific manner (Jones *et al*., 2007; Steinmann *et al*., 2007). Recently, it was confirmed that p7 is not incorporated into the virion, but is critical for capsid assembly and envelopment (Gentzsch *et al*., 2013; Vieyres *et al*., 2013).

NS2 is a 23kDa transmembrane protein, anchored in the ER. NS2 itself is not required for HCV RNA replication, but together with NS3, it consititutes the NS2/3 protease, processing

the cleavage between NS2 and NS3 (Hijikata *et al*., 1993), which is crucial for viral replication (Welbourn *et al*., 2005; Lackner *et al*., 2004). It was found that mutations in NS2 active site dramatically impaired RNA replication (Jones *et al*., 2007). A growing number of evidences have suggested the important role of NS2 in the production of infectious virus. It serves as a scaffold for virus assembly by interacting with both HCV structural and nonstructural proteins, including E1, E2, p7, NS3, and NS5A (Yi *et al*., 2007; Ma *et al*., 2011). Moreover, mutations in the C-terminus of NS2 have been found to disrupt a maturation step that is required for converting a viral assembly intermediate to a fully infectious virus particle (Yi *et al*., 2009).

NS3 is a 67kDa protein, possessing two domains with multiple enzymatic activities. Its N-terminus carries a serine protease activity and the C-terminus has a helicase/NTPase activity (Gallinari *et al*., 1998). NS4A is a 8kDa protein that interacts with the protease domain of NS3. NS4A mainly functions as a cofactor for NS3 protease activity (Failla *et al*., 1994). During HCV polyprotein processing, the protease complex NS3/4A respectively cleaves the NS3-NS4A, NS4A-NS4B, NS4B-NS5A and NS5A-NS5B junctions, generating viral NS proteins (Bartenschlager *et al*., 1993; Bartenschlager *et al*., 1994). NS3/4A can also cleave some cellular factors to antagonize several host innate immune factors (Sumpter *et al*., 2005). Indeed, it is well established that NS3/4A cleaves MAVS (mitochondrial antiviral signaling, MAVS, also known as IPS-1, VISA and CARDIF) and TRIF (Toll–interleukin-1 receptor domain–containing adaptor inducing IFN-β), two proteins that are crucial for the induction of host IFN response (Foy *et al*., 2005; Li *et al*., 2005; Cheng *et al*., 2006). Due to the important role of NS3/4A protease in the viral lifecycle and its blocking in host IFN signaling response, HCV NS3/4A represents a potent target for designing DAAs. Inhibitors of NS3/4A were indeed the first DAAs to be used in HCV treatment.

NS4B is a 27kDa hydrophobic protein, harboring at least four transmembrane domains and N- and C-cytoplasmic termini (Lundin *et al*., 2003). By using GFP-NS4B fusion protein, it was found that NS4B protein localizes in the ER and membrane-associated foci (MAFs) (Gretton *et al*., 2005). Moreover, fluorescence recovery after photobleaching (FRAP) experiments showed that NS4B has slower mobility in MAFs than in the ER, suggesting different activities for NS4B in these two locations. NS4B is mainly located at ER and induces an alternation of the ER membranes, called “membranous web”, where NS4B together with

other HCV nonstructural proteins form the replication complexes (RCs) (Egger *et al*., 2002; Gretton *et al*., 2005). It was firstly considered that viral RNA replication occurs in these membrane-rich subcellular compartments. However, in the presence of HCV core protein, viral RNA was also synthesized at the proximity of lipid droplets (LDs), because core recruits biologically active RCs to LDs (Miyanari *et al*., 2007).

NS5A is a membrane-anchored phosphoprotein with three functional domains (Fig.5). Domains I and II are required for HCV RNA replication, while domain III is found to be dispensible for viral RNA replication but required for the assembly of infectious viral particles. Particularly, the serine cluster in domain III, by interacting with core, plays a pivotal role in regulating the early steps of HCV particle formation (Shi *et al*., 2002; Penin *et al*., 2004; Appel *et al*., 2005; Schaller *et al*., 2007; Appel *et al*., 2007; Appel *et al*., 2008; Masaki *et al*., 2008; Hughes *et al*., 2009a; Hughes *et al*., 2009b). NS5B has two phospho-isoforms, basally phosphorylated p56 (56kDa) and hyperphosphorylated p58 (58kDa). A number of cell culture-adaptive mutations have been mapped to NS5A, these mutations were shown to affect NS5A hyperphosphorylation, suggesting that phosphorylation status of NS5A has an influence on RNA replication efficiency (Krieger *et al*., 2001; Lohmann *et al*., 2001). NS5A can also affect RNA replication by binding to HCV NS5B and regulating its polymerase activity. In fact, NS5A is thought to be the most excursive viral protein of HCV. By interacting with many viral and host proteins, NS5A is involved in viral RNA replication, viral particle assembly, IFN resistance, and multiple cell pathways (He *et al*., 2001; Tan & Katze, 2001; Macdonald *et al*., 2004; Gale *et al*., 2004; Gale & Foy, 2005; He *et al*., 2006; Benga *et al*., 2009). Importantly, the interaction of NS5A with cellular protein p53, which inhibits p53-mediated apoptosis, is believed to promote the progression of HCC (Lan *et al*., 2002). Owing to its critical participation in viral replication and assembly, some DAAs targeting NS5A are under development (Cordek *et al*., 2011; Pawlotsky, 2013).

NS5B is a 65kDa protein anchored to ER membrane by the transmembrane domain located at its C-terminus. NS5B mainly functions as a RNA-dependent RNA polymerase (RdRp), which synthesizes nascent RNA using viral RNA as a template and nucleotide triphosphate (NTPs) as substrates (Zhong *et al*., 2000a; Zhong *et al*., 2000b). However, due to the poor error-prone activity of NS5B RdRp and its high replication efficiency (Behrens *et al*., 1996), HCV exhibits a great genomic heterogeneity, circulating in the blood as “quasispecies”, which

helps virus escape from host immune response and causes rapid drug resistance (Nadal *et al*., 2002; Cruz-Rivera *et al*., 2012; Domingo *et al*., 2013). Diverse interactions of NS5B with other viral proteins (NS2, NS3, NS4A, and core protein), and also cellular proteins including nucleolin, vesicle membrane protein hVAP-33 were demonstrated to be necessary for viral replication (Ishido *et al*.,1998; Shirota *et al*., 2002; Dimitrova *et al*., 2003; Hirano *et al*., 2003; Gao *et al*., 2004; Jennings *et al*., 2008). NS5B is the pivotal player in HCV replication machinery, since only HCV 2a isolate JFH1 can efficiently replicates both *in vitro* and *in vivo*, which is thought to be contributed by the RdRp (Schmitt *et al*., 2001; Simister *et al*., 2009). Given the key role of this enzyme in viral replication, NS5B has been a premier DAA therapeutic target (Membreno & Lawitz, 2011). Small molecules disrupting its function are extensively studied. Nucleoside inhibitors (NIs) function through terminating the incorporation of NTPs after they are incorporated into the RNA chain. Compounds of another class are Non-nucleoside inhibitors (NNIs), which indeed act as allosteric inhibitors, reducing RNA synthesis through catalytic inhibition rather than substrate competition (De Francesco & Rice, 2003; Ma *et al*., 2005; Koch & Narjes, 2007). Notably, any single mutation in NS5B is sufficient to cause viral resistance to these inhibitors (Migliaccio *et al*., 2003; Olsen *et al*., 2004; Tomei *et al*., 2004; Paolucci *et al*., 2013).

4.3 The HCV life cycle

As an RNA virus, the life cycle of HCV is entirely cytoplasmic. It starts with the entry of HCV into hepatocytes via receptor-mediated endocytosis (Fig.7 step1). Following virus uncoating, the viral genome is released into the cytoplasm and instantly translated through HCV IRES-mediated translation at the ER, producing a single polyprotein precursor. This protein precursor is co- or post-translationally processed by cellular and viral proteases into mature nonstructural and structual proteins (Fig.7 step2 and step3). In the detergent-resistant lipid rafts, viral NS proteins NS3-NS5 form the replication complexes in which NS5B RdRp directs the synthesis of progeny genomic RNA after the production of a negative strand RNA template (Fig.7 step4) (Shi *et al*., 2003; Aizaki *et al*., 2004). Newly synthesized positive-strand RNAs are encapsidated by mature core protein which homodimerizes and is trafficked to the LDs. In the proximity of the LDs, viral particles assemble by recruitment of HCV envelope proteins E1-E2 complexes and budding into ER (Fig.7 step5). In the ER, viral particles undergo maturation through interacting with cellular lipoproteins (Fig.7 step6).

Mature viral particles exit the infected cell through p7-buffered secretion with the characteristic of low buoyant density (Lindenbach & Rice, 2013).

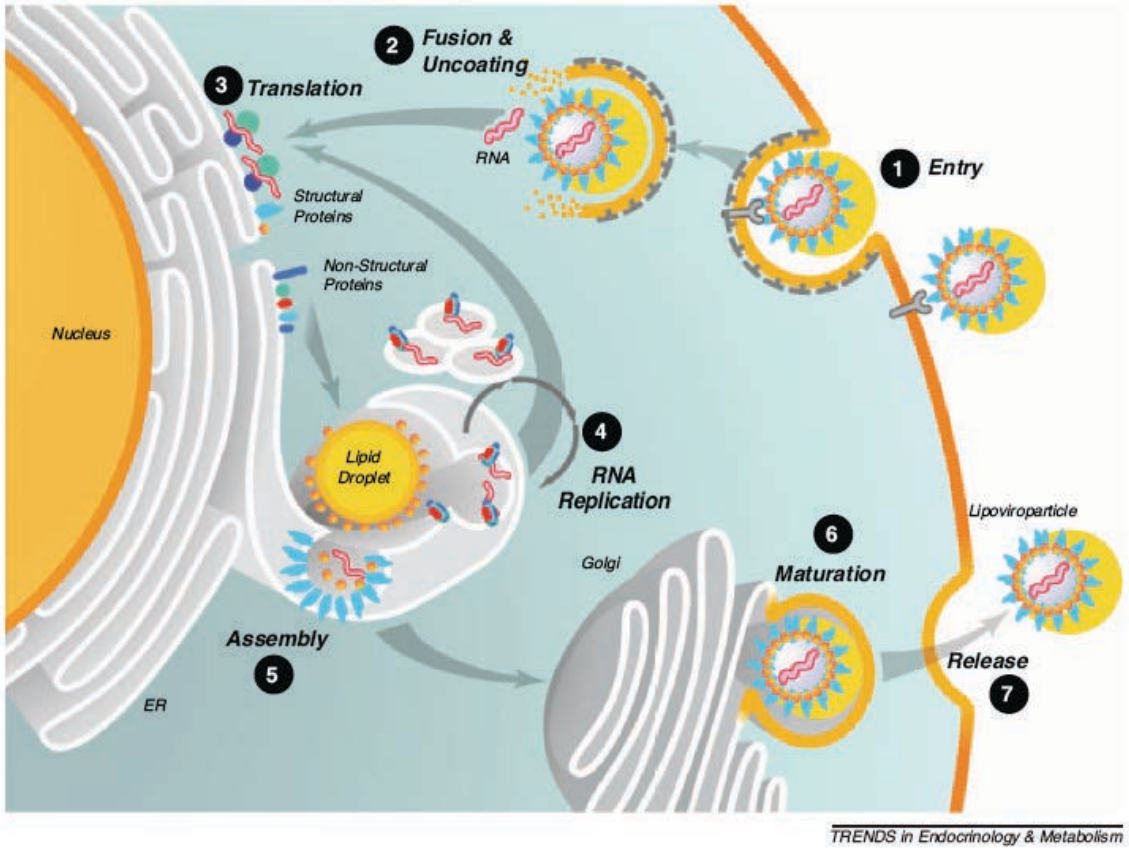


Fig. 7: Schematic representation of the HCV life cycle
(Herker & Ott, 2011)

Throughout the whole viral lifecycle, HCV overturns a variety of cellular pathways and activities to facilitate many aspects of its multiplication. Using a genome-wide siRNA library, many host factors that support HCV replication have been identified (Tai *et al*., 2009). Conversely, by microarray analysis of gene expression profile of HCV JFH1 infected-Huh7 cells, it is observed that the expression of host genes involved in cellular defense mechanisms (including inflammation, proliferation, apoptosis, and antioxidant responses), intracellular transport (including cytoskeleton regulation and vesicle trafficking) and cellular metabolism (including protein and lipid metabolism) was significantly altered after HCV infection (Blackhaam *et al.*, 2010). Extensive studies have demonstrated that the HCV life cycle is tightly linked to cellular lipid metabolism. The interaction between HCV and cellular lipids starts soon after viral protein synthesis in the infected cells. The transfer of mature form of HCV core from the ER to the LDs is an important step for the subsequent viral assembly (Shavinskaya *et al*., 2007). Trafficking of

core to LDs gives rise to the redistribution of intracellular LDs due to the replacement of adipocyte differentiation-related protein (ADRP) by core on the surface of LDs (Boulant *et al*., 2008). Importantly, preventing the LDs redistribution through disrupting the microtubule network or by using anti-dynein antibodies to block dynein motor protein leads to reduced virus production (Boulant *et al*., 2008). It was proposed that core-induced redistribution of LDs at the nuclear periphery, increased the probability of interaction between RNA replication factories and the sites of virion assembly, thus it might be an important process for HCV switching its RNA replication to virus assembly. This hypothesis is consistent with the observation that in the ER-derived membranous web, sites that contain replicating dsRNA are often in the vicinity of LDs with core attached on the surfaces (Fig.8). Host Diacylglycerol Acyltransferase 1 (DGAT1) was identified as a key factor required for HCV particle formation. It binds HCV core and localizes core to the DGAT1-generated LDs, further supporting the hypothesis that the production of infectious HCV particles does not randomly occur in close proximity of the ceulluar LDs (Herker & Ott, 2011). In the absence of core, no such close spacial association between LDs and sites of RNA synthesis was found (Targett-Adamas *et al*., 2008a; Targett-Adamas *et al*., 2008b). However, a recent study showed that this spacial association can be also established in HCV replicon cells in which viral structural proteins, notably core protein is absent (Vogt *et al*., 2013).

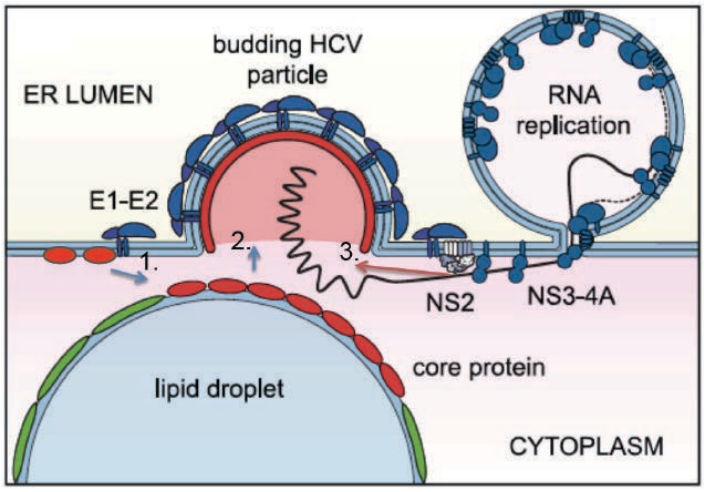


Fig. 8: Lipid droplets in the early stage of virion assembly.
Adapted from (Counihan *et al.*, 2011)

Core is targeted on the surface of LDs after its synthesis and maturion on ER (arrow bar 1). At the LD/ER interface, LD-associated core is trafficked from the LDs into the nascent viral particle (arrow bar 2), simultaneously, viral RNA is recruited from the replication complex (arrow bar 3). Meanwhile, nascent viral particle buds into the ER lumen. The interaction between NS2 and NS3/4A is important for the egress of core from the surface of LDs, and also could be a signal to switch genome RNAs from replication to packaging.

In addition to induding LD redistribution, HCV core also regulates cellular lipid metabolism through different pathways (Fig.9), which is not only the requirement for completing viral life cycle but also the molecular mechanism of HCV-related steatosis and carcinogenesis (Okuda *et al*., 2002; Lerat *et al*., 2002; Perlemuter *et al*., 2002; Sabile *et al*., 2002; Moriishi *et al*., 2007; Tanaka *et al*., 2008; Khan *et al*., 2010; David *et al*., 2012). Besides HCV core protein, almost all viral proteins interact with host factors to carry out the HCV life cycle (Tellinghuisen & Rice, 2002; Castera *et al*., 2005; Shulla & Randall, 2012). It is also believed that these viral-host interactions contribute to HCV-related diseases such as inflammation, steatosis, fibrosis, disordered metabolism, insulin resistance, and hepatocellular carcinoma (HCC) (Kawaguchi *et al*., 2004; Shitani *et al*., 2004; Street *et al*., 2005; Asselah *et al*., 2006; Tanaka *et al*., 2008; Parvaiz *et al*., 2011; Badar *et al*., 2012; Amako *et al*., 2013).

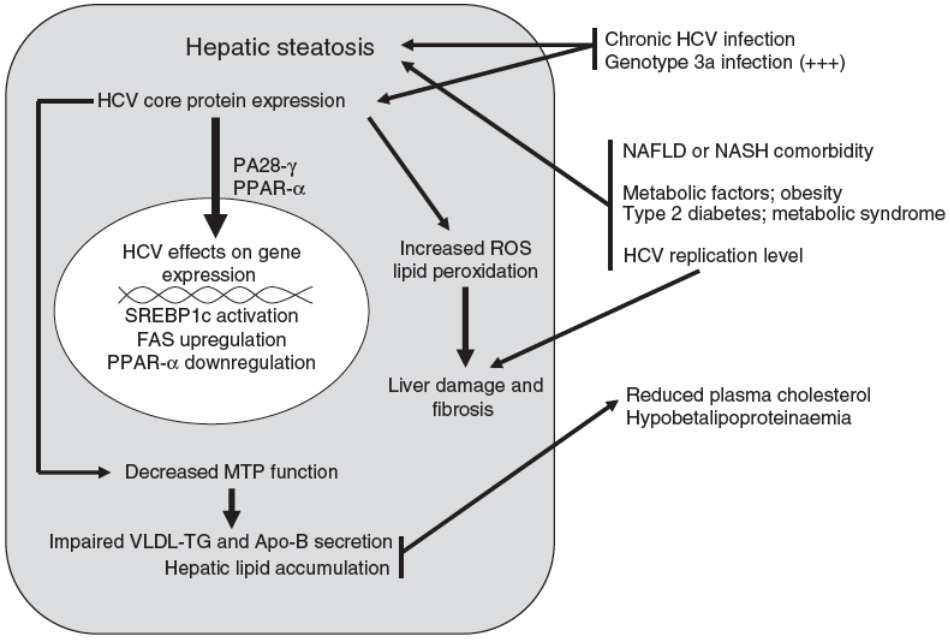


Fig. 9: Cellular lipid metabolism altered by HCV infection (Negro & Sanyal, 2009)

Of note, interactions among viral non-structural and structural protein are affirmatively required for the execution of viral life cycle (Dimitrova *et al*., 2003; Ma *et al*., 2011; Popescu *et al*., 2011). Particularly, a series of interactions between non-structural proteins and core drive the progress of HCV assembly and egress. It was demonstrated that the interaction between NS5A and LD-bound core is a first key step in HCV assembly (Appel *et al*., 2008; Masaki *et al*., 2008). Also, the interaction between NS2 and membrane-bound P7 plays an important role in organizing other NS proteins and core-containing LDs together to the

potential sites of HCV assembly (Jirasko *et al*., 2010; Popescu *et al*., 2011). Then, the interaction of NS2-P7 with NS3/4 enzyme was shown to be required for the retrieval of the LD-bound core into nascent viral particles. Interestingly, it was found that mutations in NS3 helicase domain can overcome viral assembly defects caused by genetic mutations in other NS proteins and core protein (Jirasko *et al*., 2010; Ma *et al*., 2008; Jones *et al*., 2011). Moreover, individual adaptive mutations in HCV core, NS2, NS3 and NS5A were shown to greatly enhance the physical interactions among viral structural and NS proteins, promoting the assembly of infectious viral particles (Jiang & Luo, 2012).

4.4 HCV particles

In patients, HCV particles circulating in the blood display a heterogeneity of buoyant density ranging from 1.03g/ml to 1.25g/ml, which also varies from one patient to another (Hijikata *et al*.,1993; Petit *et al*., 2005; Nielsen *et al*., 2006). The infectivity of HCV particles was found to be inversely correlated with their densities, with low-density virus being more infectious than high-density virus (Bradley *et al*., 1991; Hijikata *et al*., 1993). Low-density (<1.063g/ml) particles have been suggested to be associated with serum-derived low density lipoproteins as they can be precipitated by anti-ApoB sera, while high-density (>1.21g/ml) particles were supposed to be complexed with either high-density lipoprotein or immunoglobulins (Choo *et al*., 1995; Thomssen *et al*., 1992; Thomssen *et al*., 1993; Kanto *et al*., 1995; Pumeechockchai *et al*., 2002).

André *et al* quantified HCV RNA in the low-density fractions corresponding to the fractions of very low density lipoproteins, intermediate-density lipoproteins and low-density lipoproteins (VLDL, IDL, LDL). They found that all patients had HCV RNA in at least one of the three fractions, and HCV RNA always can be co-purified with immunoglobulins by protein A-coated magnetic beads. These immuno-purified HCV RNA-containing particles were then termed lipo-viro-particles (LVPs) (André *et al*., 2002). Characterizing the biochemical properties of the purified LVPs and VLDLs from the very low density or low density fractions, it was found that purified LVPs from both fractions contained more triglyceride per ApoB molecule than lipoproteins from the same fraction. This difference in lipid composition implied that LVPs are not just HCV virions attached to lipoproteins. Under electron microscopy, purified LVPs displayed large spherical structures with an average

diameter of 100 nm and not just classical flavivirus-like virions bound to lipoproteins, which was suggested in previous studies (Thomssen *et al*., 1992 Thomssen *et al*., 1993) After delipidation, purified LVPs appeared as capsid-like particles with the largest structures being 30-35nm in diameter, and HCV core protein in these particles were identified by a monoclonal antibody recognizing the N-terminus of core (André *et al*., 2002).

Apart from viral structural proteins including HCV core and envelope glycoproteins, immunocapture studies performed by different groups demonstrated that LVPs also contain cellular apolipoproteins such as apoB, apoE, apoC1, CII and CIII (André *et al.*, 2002; Meunier *et al*., 2008; Nielsen *et al*., 2004; Chang *et al*., 2007; Diaz *et al*., 2006; Maillard *et al*., 2006). Importantly, HCV particles from the infected patient's liver macerate can be immunoprecipitated by anti-human apoB-100 polyclonal antibodies, suggesting that the association of HCV with apoB-containing lipoproteins occurs in the hepatocytes (Nielsen *et al*., 2004). However, apoB-48, a non-exchangeable apolipoprotein exclusively generated by the small intestine cells was found to be equally present in the purified LVPs as well as apoB-100, which is an internal component of hepatic VLDLs. This puzzling observation suggested that HCV particles in the circulation originate not only from the liver but could also derive from the intestine (Diaz *et al*., 2006). To some degree, this doubt was resolved by the study of Felmlee *et al*. Their findings showed that dietary triglyceride significantly altered the buoyant density distibution of circulating HCV particles. The rapid increase of HCV RNA in the very low density fraction, which includes both the apoB-100 and apoB-48-associated particles, is probably the result of both the *de novo* production from infected hepatocytes and the intravascular transfer from the high density fraction. While they did not exclude the possibilty that apoB-48-associated HCV particles may be secreted by the intestine cells. This study demonstrates that the buoyant density of HCV particles is not only heterogeneous, but also dynamic, and depends on the triglyceride-rich lipoproteins (TRLs) in the circulation (Felmlee *et al*., 2010). LVPs circulating in patients are characterized by the presence of HCV RNA, viral envelope proteins, apolipoproteins characteristic of TRLs and high lipid contents. Normally, only one apoB molecule per TRL particle is expressed. Strikingly, in some patients the LVPs fractions contain large amounts of apoB, with more than 10^6 apoB molecules per HCV RNA genome. Such high ratio indicate that in these patients most LVPs are nucleocapsids-free, but envelope proteins and lipoproteins-containing subviral particles, so–called empty LVPs (eLVPs) (Scholtes *et al*., 2012). This is consistent with the observation

that the buoyant density of HCV particles in the plasma varies widely in patients. The presence of apoB-negative particles were also observed, although in previous study all HCV RNA from patients' plasma were immunoprecipitated by anti-apoB antibodies (Thomssen *et al*., 1992; Scholtes *et al*., 2012). So far, our knowledge on circulating HCV particles indicates that different populations of viral particles exist. All these viral particle populations in the circulation may differently contribute to the physiopathology of HCV-related extrahepatic manifestations.

Interestingly, nucleocapsids-free subviral particles can also be generated *in vitro*. Transfecting polarized human intestinal Caco-2 cell line just with HCV genes encoding envelope proteins E1 and E2 leads to the assembly and secretion of low-density hybrid particles resembling TRL and exposing viral glycoproteins on the surface. These secreted E1-E2 containing particles were only detected in apoB-containing density fractions and they can be co-immunoprecipitated by anti-apoB and anti-E2 specific antibodies. Similarly, E1-E2 and apoB containing particles were secreted from hepatoma cell line HepG2, but not by Huh7 nor Huh7.5 (Icard *et al*., 2009). This could be explained by the deficiency in lipoprotein assembly of Huh7 and Huh7-derived cell lines (Icard *et al*., 2009; Meex *et al*., 2011). Also, it is consistent with the findings that anti-apoB specific antibodies are inefficient to capture HCVcc particles, which is produced from Huh7 and Huh7-derived cells (Fig.13) (Huang *et al*., 2007; Owen *et al*., 2009; Merz *et al*., 2010). However, a recent study showed that VLDL-producing and HCV replicating HepG2 cells as well as VLDL-deficient Huh7.5 HCV replicating cells secrete mainly apoE-postive/apoB-negative viral particles, suggesting that the association of HCV virion with apoB does not necessarily depend on the ability of cells to produce VLDL *in vitro* (Jammart *et al*., 2013). In contrast to apoB, another VLDL-containing apolipoprotein, apoE, was proved to be the cellular protein incorporated into infectious HCV particles and located on the surface of HCV envelope (Chang *et al*., 2007; Cun *et al*., 2009; Jiang & Luo, 2009). Furthermore, apolipoprotein content analysis of affinity-purified HCV particles showed that each particle bears around 300 molecules of apoE at the surface (Merz *et al*., 2010). This observation indicates a remarkable enrichment of apoE in viral particles.

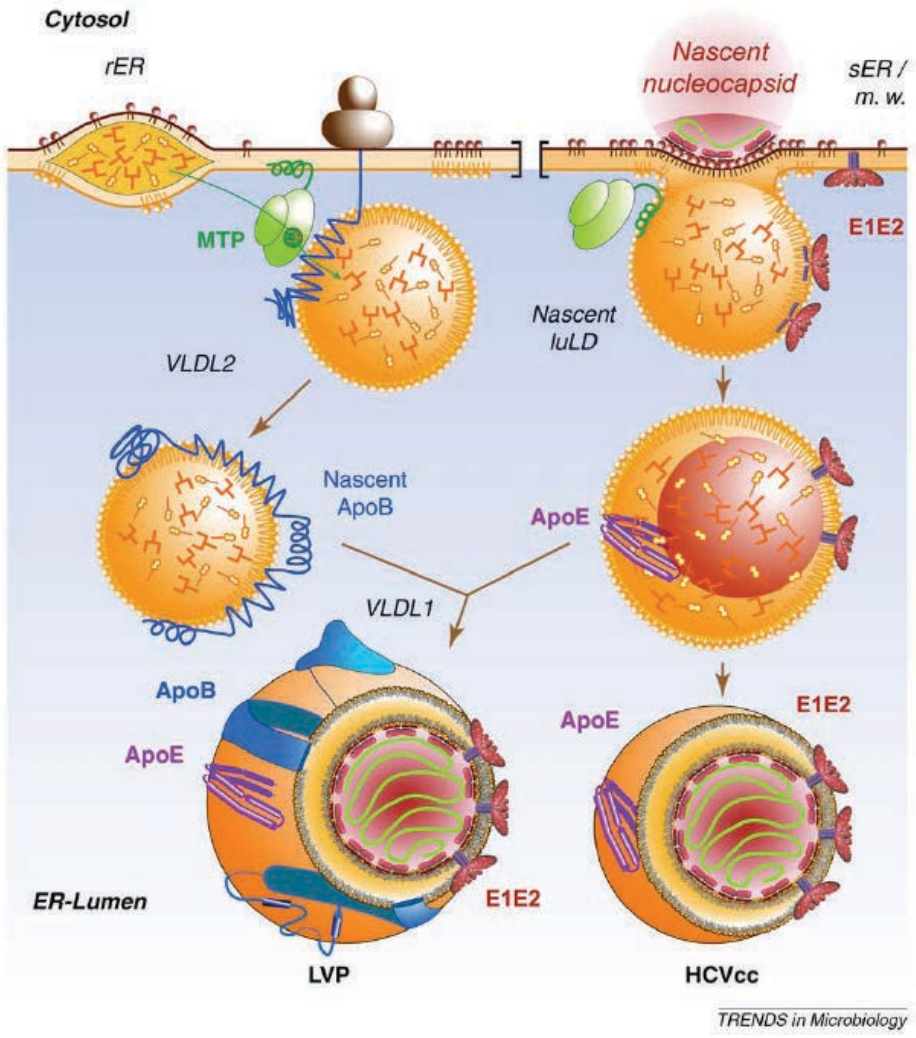


Fig.10: Models of HCV particles structure and their biogenesis.
(Bartenschlager *et al*., 2011)

Importantly, the intracellular infectious HCV particles are similar in size as secreted particles but show higher buoyant density (Gastaminza *et al*., 2006), indicating that the composition of infectious HCV particle is altered during viral egress. Indeed, HCV particles hijack the VLDL assembly and secretion pathway to be released from the hepatocytes (Huang *et al*., 2007; Gastaminza *et al*., 2008). To precisely characterize the ultrastructure of HCV virions, Rice's group also grew HCV particles from human fetal liver cells (HFLCs) which are polarized, and more physiological than hepatoma cells, thus may better reconstitute the process of *in vivo* lipoprotein and virus assembly. Their cryoelectron tomography (cryo-ET) studies showed that HCV particles generated from both Huh7.5.1 cells and HFLCs displayed cellular lipoproteins apoB and apoA-I on the virion surface, in addition to apoE. Furthermore, apoE-specific antibodies were superior than antibodies against HCV glycoproteins at capturing HCV RNA-

containing particles, suggesting either lower abundance of HCV envelope proteins or masking by apoE on the surface of viral particles (Catanese *et al*., 2013b). Thus, high abundance of apoE component in viral particles could give rise to the question that HCV associating with apoE may change the biochemical properties of HCV particles. Comparing HCVcc produced from Huh7.5 cells and from apoE-knockout Huh7.5 cells in an iodixanol density gradient assay, there was no difference between these two populations of viral particles, indicating that the association with apoE does not cause evident physical change of HCV particles. But, detailed physical and biochemical changes of HCV cannot be ruled out, as the researchers did not analyse the lipid contents, the status of HCV envelope proteins and so on, while the infectivity of viral particles generated from apoE-depleted cells was remarkably reduced (Hishiki *et al*., 2010).

Besides the enrichment of apoE incorporated in the virion, mature HCV particles were also abundant in cholesterol as tested by the molar ratio of cholesterol to phospholipid in the virion compared to that of cell membranes. Virion-associated cholesterol and sphingolipid were demonstrated to be required for HCV infection, as shown by cholesterol depletion with MßCD or by sphingomyelin (SM) hydrolysis with SMase (Aizaki *et al*., 2008; Yamamoto *et al*., 2011). Two hypothesis could explain why mature HCV particles are rich in cholesterol. A large proportion of HCV structural proteins are found to partition into cellular detergent-resistant membranes (DRMs) (Matto *et al*., 2004; Aizaki *et al*., 2008), which are ER-derived lipid raft-like membranes. Also, virion assembly is thought to take place at the sites in ER membranes in close proximity of the LDs (Miyanari *et al*., 2007). LDs are the cellular organelles storing neutral lipids such as triglycerides and cholesterol esters. The neutral lipids constitute the core of LDs and are surrounded by an outer layer composed by amphipathic lipids including phospholipids and cholesterol (Brown, 2001; Martin & Parton, 2006). Thus, these cholesterol- and sphingolipid-rich microdomains in the membrane compartments may be involved in HCV virion maturation. Another explanation for the recruitment of these lipids to HCV virion membrane may be due to the tight link between virus production and VLDL metabolism in HCV-infected hepatocytes (Huang *et al*., 2007).

4.5 HCV model system

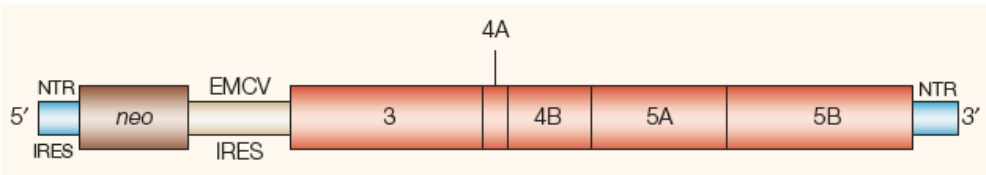
After the discovery of HCV in 1989, research on HCV has been impeded for a long time due to the lack of a robust infection-replication system. Classical virological methods were not able to establish productive HCV infection *in vitro*. However, with the help of new molecular cloning techniques, researchers were capable of developing several HCV cellular model systems to investigate various aspects of the HCV life cycle.

4.5.1 HCV cellular models

HCV replicon system

The subgenomic replicon system, in which HCV subgenomic RNA efficiently replicates in human hepatoma cell lines (Lohmann *et al*., 1999), was the first milestone in the studies of HCV RNA replication, and the interactions between viral proteins and the host cell (Blight *et al*., 2000; Krieger *et al*., 2001; Blight *et al*., 2002; Bukh *et al*., 2002; Evans *et al*., 2004; Zhang *et al*., 2004; Hamamoto *et al*., 2005; Korf *et al*., 2005; Li *et al*., 2005; Wang *et al*., 2005; Kaul *et al*., 2009). Moreover, old subgenomic and additionally developed genomic replicon systems provided a potent tool to screen small anti-HCV molecules, to identify and analyse drug-resistant mutations, replication enhancing mutations and cell-culture adaptive mutations, and also to investigate HCV highly permissive cell lines (Frese *et al*., 2002; Bartenschlager, 2002; Bartenschlager & Pietschmann, 2005; Tong & Malcolm, 2006; Kim *et al*., 2007; Yi *et al*, 2007; Steinmann & Pietschmann, 2005). Although HCV full-length genome can persistently replicate and express all the viral proteins, there is no production of viral particles (Pietschmann *et al*., 2002; Date *et al*., 2007), and HCV entry and assembly can not be investigated with these genomic replicon systems.

a.



b.

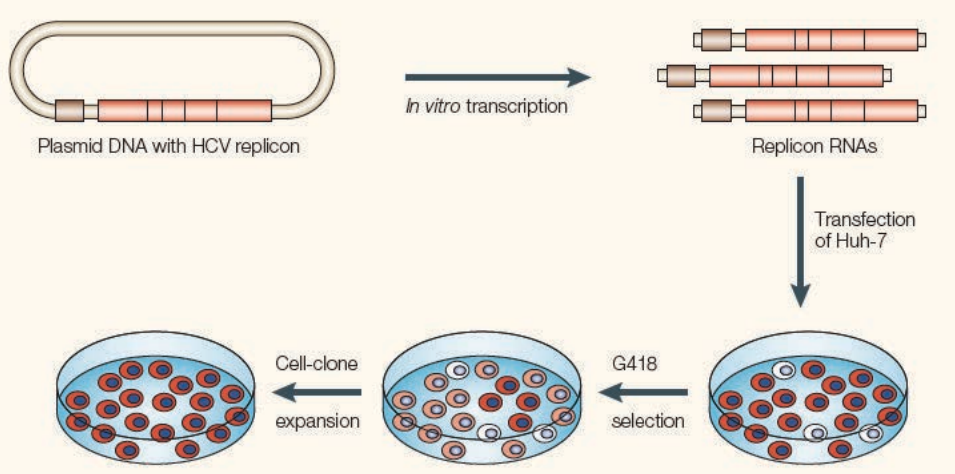


Fig. 11: Schematic representation of HCV subgenomic replicon (a) and establisment of HCV replicon system (b) (Bartenschlager, 2002).

a. HCV genomic region from core to NS2 was replaced by neomycin phosphotransferase gene (neo) and the internal ribosom-entry site (IRES) of encephalomyocarditis virus (EMCV). The translation of selectable marker neo is directed by HCV IRES which is in the 5'NTR, while the expression of HCV replication proteins N3-NS5B is under the control of EMCV IRES.

b. HCV replicon RNAs were *in vitro* transcribed from DNA plasmids carrying HCV subgenome, and they were introduced into human hepatoma cell line Huh7 by transfection. Cells were cultured in medium containing G418 to select G418-resistant clones, in which HCV replicons self-amplify and express the neomycin phosphotransferase to inactivate G418. The postive colonies were isolated and grown to form a cell clone which carries stably replicating HCV subgenomic replicons.

HCVpp sytem

During the same period, HCV pseudoparticles (HCVpp) system was developed by several groups to better understand HCV entry process (Bartosch *et al*., 2003a; Drummer *et al.*, 2003; Hsu *et al*., 2003). HCVpp are recombinant viral particles that consist of retroviral or lentiviral core proteins surrounded by an envelope where HCV envelope glycoproteins are incorporated and present on the surface. HCVpp were generated by co-transfection HEK-293T cells with three plasmid constructs (Fig.12): HCV E1E2 expressing construct, a provirus construct expressing a reporter gene such as Luciferase (Luc) or green fluorescent protein (GFP), and a construct encoding HIV (Human Immunodeficiency Virus) or MLV (Murine Leukemia Virus) gag-pol proteins (Bartosch *et al*., 2003a; Hsu *et al*., 2003). Recombinant viral particles are harvested from the supernatant, and after purification they are used to infect target cells such as Huh7 or Huh7-derived Huh7.5 cells. The infectivity is measured by quantifying the

activity of the reporter gene. Importantly, the entry function of HCVpp is mediated by the functional HCV envelope glycoproteins E1 and E2. Infection of HCVpp in the hepatoma cells can be neutralized by anti-E1, anti-E2 antibodies or sera from HCV-infected patients, or anti-CD81 antibodies (Bartosch *et al*., 2003b). Furthermore, HCVpp entry is pH-dependent (Hsu *et al*., 2003), suggesting that the entry process of HCVpp to some degree is similiar to that of authentic HCV particles. Therefore, HCVpp system has been extensively used to study the mechanisms of HCV entry and to identify cellular entry factors and membrane fusion. Since its establishment, a number of cellular factors have been identified or confirmed by using this system (Ploss & Evans, 2012). In addition, HCVpp system has also been widely used to evaluate neutralizing antibodies from HCV infected-patients or experimentally infected chimpanzees (Bartosch *et al*., 2003b; Logvinoff, *et al*., 2004; von Hahn *et al*., 2007). Moreover, as HCVpp can be manufactured using envelope proteins from variable HCV isolates, this system has also been used to investigate viral tropism, and to compare the differences in the receptor interaction with diverse HCV glycoproteins (McKeating *et al*., 2004; Owsianka *et al*., 2005). Since HCVpp system can be used to study viral entry events independently of other steps of the HCV life cycle, it is a potential tool to develop antiviral compounds targeting viral entry (Baldick *et al*., 2010). Therefore, HCVpp system is quite practical in the study of HCV entry, however it has inherent limitations. As they are produced in non-liver cells (293T cells), in which the VLDL assembly pathway is deficient, HCVpp cannot be used to study the role of lipoproteins in HCV entry. Indeed, HCV viral RNA replication and virion assembly highly depend on cellular lipid and cholesterol metabolism (Ye *et al*., 2003; Chang *et al*., 2007; Ye, 2007; Popescu *et al*., 2011), and viral particles are co-assembled with VLDL in the hepatocytes, leading to the formation of unique lipo-viro-particles (Huang *et al*., 2007). However, the development of the HCVpp system was a real breakthrough to study the function of HCV envelope glycoproteins in virus entry since before the establisment of HCVpp system, models primarily used for studying HCV entry were based on recombinant HCV envelope glycoproteins and the HCV-like particles (Baumert *et al*., 1998; Blanchard *et al*., 2002).

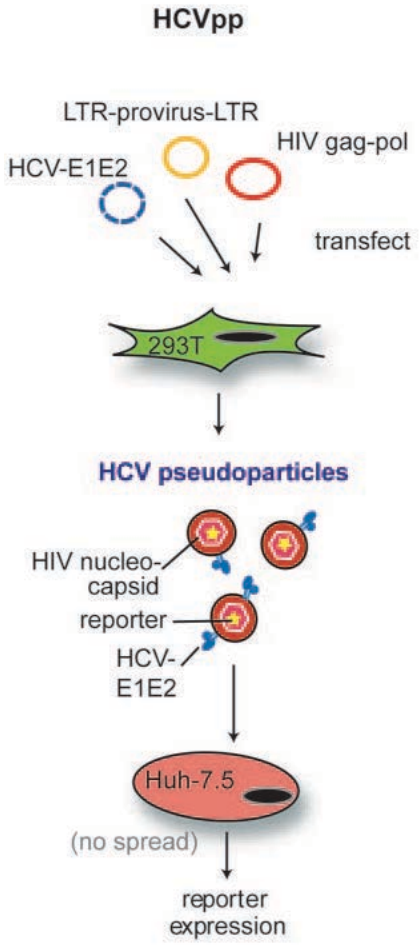


Fig. 12: Schematic representation of HCVpp system
(Tellinghuisen *et al*., 2007)

HCVcc sytem

The most important breakthrough in the history of HCV *in vitro* models is the development of an infection and replication competent cell culture (HCVcc) system (Cai *et al*., 2005; Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al*., 2005). In this system, only HCV genotype 2a strain JFH1 can efficiently replicate in cell culture without the requirement for adaptive mutations, and it can reliably produce infectious viral particles (Zhong *et al*., 2005). Consequently, HCVcc system addressed the limitation of HCV replicon and HCVpp systems, and it can be used to investigate all stages of HCV lifecycle. Firstly, results obtained with HCVpp or HCV replicon system were confirmed in HCVcc system. For instance, cellular factors such as CD81, scavenger receptor SRB1, CLDN1 and OCLN have been found as essential entry molecules initially by using a soluble form of E2 (sE2) or HCVpp, then by using HCVcc, their role in virus entry could be validated (Pileri *et al*., 1998; Scarselli *et al*.,

2002; Evans *et al*., 2007; Ploss *et al*., 2009). Also, pH-dependent entry of HCVpp and clathrin-mediated endocytosis were verified by using HCVcc (Blanchard *et al*., 2006; Codran *et al*., 2006). In terms of viral RNA replication, the involvement of cellular lipids in HCV replication observed with replicon system was further confirmed in the HCVcc system (Ye *et al*., 2003; Kapadia & Chisari, 2005; Huang *et al*., 2007). Furthermore, the dependence of RNA replication on host factor cyclophilins was found both in the replicon and HCVcc system (Robida *et al*., 2007; Yang *et al*., 2008). Importantly, the late steps of the HCV life cycle such as virus assembly and release could not be investigated before the development of the HCVcc system. The formation of HCV-like particles were observed in insect cell using a recombinant baculovirus or in mammalian cells (BHK-21) using Semliki forest virus vectors expressing HCV structural proteins, but viral particles were not secreted in these systems (Baumert *et al*., 1998; Blanchard *et al*., 2002). In contrast, infectious particles are assembled and secreted in HCVcc system, and some mutants can yield high viral titers (Cai *et al*., 2005; Zhong *et al*., 2006; Kaul *et al*., 2007; Koutsoudakis *et al*., 2007; Pokrovskii *et al*., 2011). Moreover, replacing the sequence Core-NS2 of JFH1 with that from other genotypes or subgenotypes, a panel of JFH1-based chimeric viruses containing the structural proteins from genotype 1 to 7 could be generated from hepatoma-derived Huh7/Huh7.5 cells after they are electroparated with *in vitro* synthesized HCV RNA (Fig.13) (Lindenbach *et al*., 2005; Pietschmann *et al*., 2006; Gottwein *et al*., 2007; Yi *et al*., 2007). These viruses were then used for comparative studies of antivirals, receptor interactions, cross-genotype neutralization, and HCV genotype-specific functional differences (Jensen *et al*., 2008; Scheel *et al*., 2008; Gottwein *et al*., 2009; Sheel *et al*., 2012). Importantly, HCVcc chimeric strain J6/JFH1 can establish long-term infection in chimpanzees and in liver-humanized mice (uPA-SCID mice transplanted with human hepatocytes) (Bukh & Purcell, 2006). Viruses grown in these infected animals can be isolated and remain highly infectious in cell culture (Fig.13). Thus, the extended utilization of HCVcc in animal models makes it feasible to study a genetically defined virus simultaneously *in vitro* and *in vivo*, allowing the mechanisms of viral pathognesis, immune escape and antibody neutralization to be well deciphered at the molecular level. Notably, the specific infectivity of virus produced by infected chimpanzees and humanized mice is much higher than that of cell-cultured HCV, and the increased specific infectivity of HCV grown *in vivo* correlated with the shift of HCV RNA-containing particles to a lower buoyant density. This difference indicated that the physical association of authentic HCV particles with low density factors influence viral infectivity (Lindenbach *et al*., 2006).

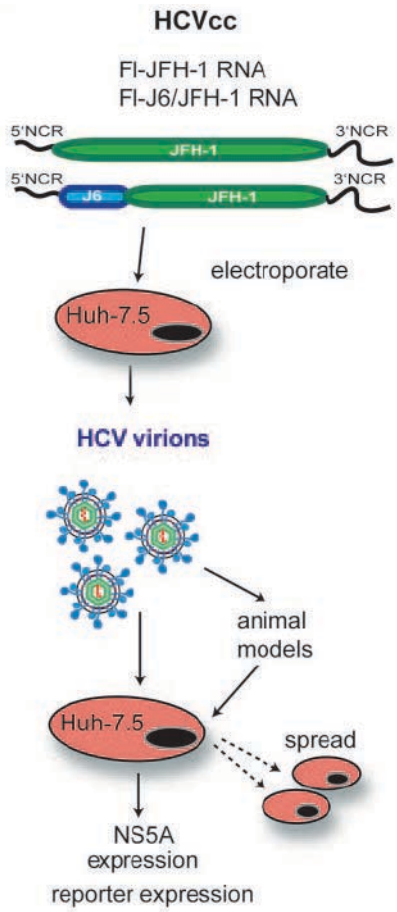


Fig.13: Schematic representation of HCVcc system
(Tellinghuisen *et al*., 2007)

In conclusion, HCV cellular models mentioned above greatly pushed forward the basic research on HCV and were essential for the development and/or validation of new antiviral drugs against HCV.

4.4.2 HCV animal models

Chimpanzees

Naturally, HCV infects only humans, and chimpanzee is the only animal model in which HCV can replicate at a high level. This primate has therefore been extensively used for studying HCV infection in the past decades. Studies of HCV in chimpanzees provided a large amount of knowledge regarding the molecular, immunological and clinical aspects of HCV

infection, even the discovery of HCV was attributed to the utilization of this animal model (Kuo *et al*., 1989; Bigger *et al.*, 2004). HCV-infected chimpanzees displayed an increased blood alanine aminotransferases (ALT) level and necroinflammatory changes determined by liver biopsies at the acute phase. These clinical characteristics were consistent with the observations in acute-infected patients. However, the chronicity rate of HCV in chimpanzees is around 30-60%, lower than that in humans, where it is up to 85% (Hoofnagle *et al*., 1997; Bassett *et al*., 1998). Besides, HCV-related liver diseases in chimpanzees are significantly milder. Chronically infected chimpanzees rarely progress to liver fibrosis or cirrhosis, and only one chimpanzee has been reported to develope HCV-associated hepatocellular carcinoma (Muchmore *et al*., 1988; Walker *et al*., 1997). Nevertheless, experimentally infected chimpanzees were quite instrumental for the study of viral kinetics, outcome of infection, host immune responses and the evaluations of anti-HCV agents, or prophylactic and therapeutic vaccination against HCV (Shimizu *et al*., 1990; Farci *et al*., 1992; Su *et al*., 2002; Puig *et al*., 2004; Barth *et al*., 2011; Olsen *et al*., 2011). Particularly, knowledge obtained from immunological studies in chimpanzees offers the theoretical basis for the development of efficacious HCV vaccine (Farci *et al*., 1994; Bassett *et al*., 2001; Thimme *et al*., 2002; Barth *et al*., 2011). In turn, different prophylactic and therapeutic vaccine candidates have been evaluated in chimpanzees before they got to the clinical trials (Folgori *et al*., 2006; Dahari *et al*., 2010).

Mouse models

Due to high cost and ethical concerns, chimpanzees are gradually being replaced by small animal models. Researchers have been devoted to engineer mice to support human hepatocyte grafts, thus to overcome the restricted species tropism of hepatitis viruses. The human hepatocytes-transplanted uPA/SCID (homozygous urokinase-type plasminogen activator/ severe combined immunodeficiency) mouse was the first-constructed and best-characterized small animal model (Fig.14), which can be infected with both HBV and HCV (Mercer *et al*., 2001; Meuleman *et al*., 2005; Utoh *et al*., 2008). Human albumin and other plasma proteins of human origin in mouse plasma are measured to evaluate the integrity and functional status of transplanted human hepatocytes (Meuleman *et al*., 2005). Indeed, there is a strong correlation between human albumin level in mouse blood and the human chimerism in mouse liver (Bissig *et al*., 2010). High human chimerism (up to 85% repopulated human hepatocytes) are required for a productive HCV infection in this mouse model (Steenbergen *et al*., 2010).

Although the SCID/Alb-uPA mice are not easy to maintain as they are severely immunodeficient, this animal model has been extensively used to evaluate different preventive and therapeutic strategies against HCV. Kneteman *et al.* treated genotype 1a HCV-infected mice with IFN-alpha, BILN-2061 (a NS3 protease inhibitor), or HCV371 (a NS5B polymerase inhibitor) and found that performance of all these antiviral drugs in the chimeric mice paralleled the responses in patients, confirming that chimeric mice can serve as a potenital model for the preclinical trials of anti-HCV treatment (Kneteman *et al.*, 2006). Indeed, the chimeric mice model has been extensively used to test antivirals against HCV, such as a novel NS5B inhibitor (HCV796), an anti-NS3/4A inhibitor (telaprevir), and a cyclophiline inhitor (DEBIO-025), and also combination therapies with these inhibitors on HCV infection of different genotypes (Inoue *et al*., 2007; Kneteman *et al*., 2009; Kamiya *et al*., 2010; Shi *et al*., 2013). Strategies targeting HCV cell entry have also been studied in the chimeric mouse model. Firstly, neutralizing antibodies purified from HCV genotype 1a infected patient blood has been shown to protect chimeric mice from infection with HCV homologous and heterologous strains (Meuleman & Leroux-Roels, 2008; Vanwolleghem *et al*., 2008; Meuleman *et al*., 2011). Neutralizing mAbs specific of HCV envelope protein E2 also exhibited broadly cross-neutalizing activity against HCV quasispecies in the uPA-SCID mouse model (Law *et al*., 2008). Furthermore, prophylactic treatment with anti-CD81 or anti-SRB1 antibodies completely protected human liver chimeric uPA-SCID mice from a subsequent challenge with different HCV genotypes (Meuleman *et al*., 2008; Meuleman *et al*., 2011; Lacek *et al*., 2012). The uPA-SCID mouse model has also been successfully used to assess the efficacy of small molecules targeting HCV entry factors including amphipathic DNA polymers and Griffithsin (Matsumura *et al*., 2009; Meuleman *et al*., 2011). Moreover, the clinically approved drug erlotinib, an EGFR inhibitor (specifically targets the epidermal growth factor receptor (EGFR) tyrosine kinase, clinically used to treat lung cancer), was shown to be able to delay initial HCV infection in the uPA-SCID mouse model (Lupberger *et al*., 2011; Qi *et al*., 2012; Kobayashi & Hagiwara, 2013). Coincidentally, ezetimibe, another medication, known as a cholesterol absorption inhibitor targeting Niemann-Pick C1-like-1 (NPC1L1) was also demonstrated to delay the establisment of HCV GT1 clinical isolate infection in the chimeric mice, confirming NPC1L1 as a HCV co-entry factor (Sweeney & Johnson, 2007; Sainz *et al*., 2012).

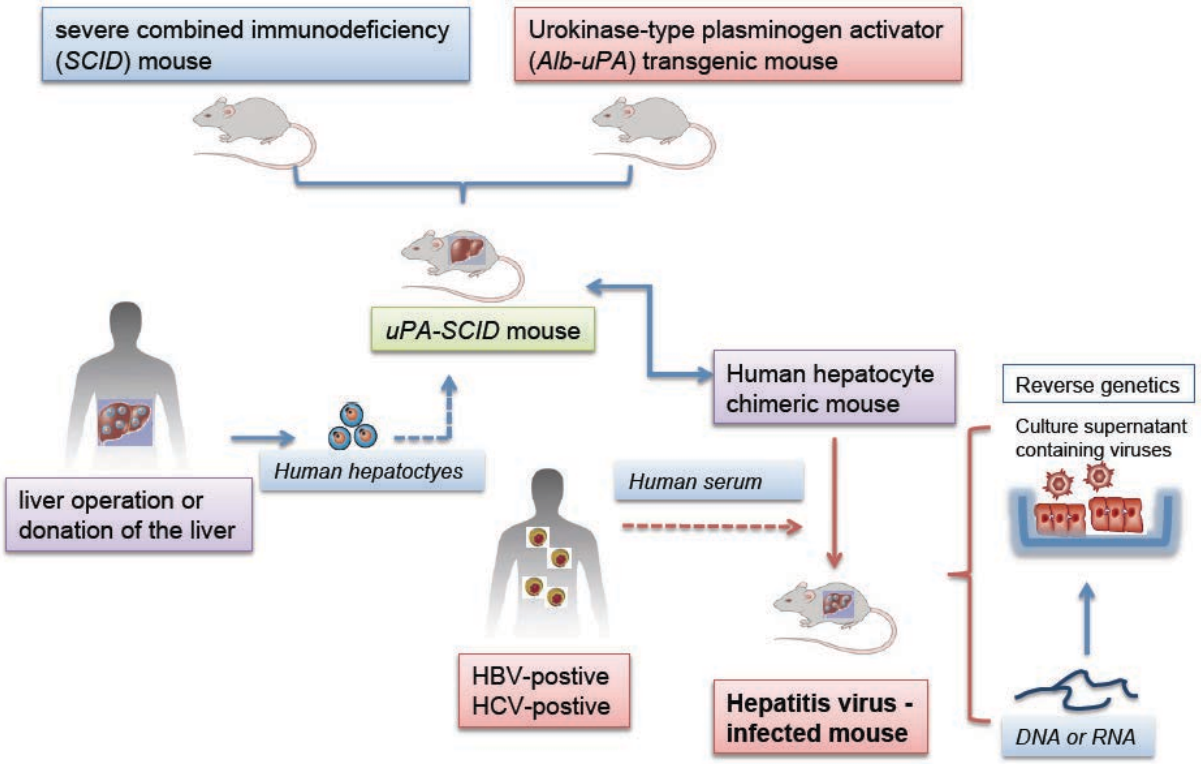


Fig.14: Schematic drawing of generation of uPA/SCID mouse model for HCV infection study. Adapted from (Chayama *et al*., 2011)

Because of its severe immunodeficiency background, the liver-humanized mouse model cannot be used to investigate host adaptive-immune responses to HCV infection or to evaluate HCV vaccines. Nevertheless, this model can be utilized to characterize HCV-induced host innate-immune responses (Walters *et al*., 2006). But, the uPA-SCID mice model has some limitations, mainly due to low breeding efficiency, a narrow window for hepatocytes transplantation and excessive mortality. To overcome these limitations, a second mouse model, the Fah-/- Rag2-/-γ-c-/- (FRG) mouse was developed. This immunodeficient strain of mice is deficient of the tyrosine catabolic enzyme fumarylacetoacetate hydrolase (Fah). Fah mutant mice develop liver disease when the protective drug NTBC (2-52-nitro-4-trifluoromethyl benzoyl)-1,3-cyclohexanedione) is withdrawn. At this time, FRG mice can be efficiently repopulated with human hepatocytes (Azuma *et al*., 2007). However the FRG mice are less extensively used than the uPA-SCID mice. They were just reported to be successfully infected with several HCV strains in one study (Bissgig *et al*., 2010). Recently, the entire HCV life cycle has been demonstrated in genetically-humanized mice, representing a breakthrough toward an immune-competent mouse model. In this mouse model some innate immune mechanisms have been

blunted and they were genetically-engineered to stably express human CD81 and OCLN (Dorner *et al*. 2013). Thus, this humanized mouse model offers the possibility for preclinical evaluation of candidate vaccines. Looking back to the journey of HCV animal models, a variety of models including callitrichidae, tree shrews, rats, transgenic mice and various mouse models have been created. The *in vivo* studies in these models have greatly contributed to our current knowledge on HCV (Meuleman & Leroux-Roels, 2009; Mailly *et al*., 2013).

II. HCV viral particle components in viral entry

1. HCV host cell tropism and transmission routes

Though human hepatocytes are the major targets of HCV, increasing evidence have shown the existence of HCV in extrahepatic organs, such as peripheral blood mononucleated cells (PBMCs) and lymphoid cells including T lymphocytes, B lymphocytes and B-cell lymphoma cells (Laskus *et al*., 1998; Sung *et al*., 2003; Bare *et al*., 2005; Kondo *et al*. 2007; Murakami *et al*., 2008; Kondo *et al*., 2011; Sarhan *et al*., 2012). The lymphotropism and direct interaction of HCV with these cells could explain the carcinogenesis of the lymphoid cells and the disorders of host immune response in HCV persistent infection (De Vita *et al*., 1995; Weng & Levy, 2003; Machida *et al*., 2005; Rosa *et al*., 2005). HCV RNA-positive PBMCs such as monocytes and dendritic cells, as well as lymphoid cells were also described as potential reservoirs that may contribute to the recurrence of HCV infection and clinically difficult-to-treat extrahepatic diseases (Barth *et al*., 2005; Coquillard & Patterson, 2009; Pham *et al*., 2008; Pham *et al*., 2010). Moreover, recent reports have shown that HCV can establish infection in neuroepithelioma-derived cell lines and endothelial cells of the blood-brain barrier (Fletcher *et al*.2010; Fletcher *et al*., 2012), which could offer a clue to the central nervous system (CNS) abnormalities in chronic HCV-infected patients (Weissenborn *et al*., 2009).

As HCV is mainly hepatotropic, substantial *in vitro* studies on HCV infection were carried out in hepatic-derived cells such as Huh7 cells and HepG2 cells. HCV initiates its infection by entry into host cells. There exists at least two routes: cell-free infection and cell-cell transmission (Valli *et al*., 2006) (Fig.17). The infection by cell-free HCV particles can be blocked by anti-E2 neutralizing antibodies or in agarose-containing culture, whereas direct transmission of virus between cells does not involve the release of viral particles into the

extracellular space, and is relatively nAbs-resistant (Witteveldt *et al*., 2009; Calland *et al*., 2012). Notably, HCV cell-to-cell transmission requires cell-cell contacts and the assembly of infectious viral particles in the donor cells, since no cell-cell transmission was observed when the donor cells were electroporated with viral RNA lacking the envelope proteins E1E2-encoding regions (Timpe *et al*., 2008; Witteveldt *et al*., 2009). These findings provided potential mechanisms that can partly explain the ineffectiveness of serum anti-HCV antibodies in controlling HCV chronic infection, in addition to the conventional genetic escape mechanism such as the "quasispecies" nature of HCV in the bloodstream and the high rate of amino acid subsitutions in the neutralization epitopes located in HCV envelope glycoproteins (von Hahn *et al*., 2007). Of note, cell-free transmission route are most likely responsible for the spread of HCV between hosts, and HCV reinfection in liver allograft patients after transplantation, while HCV cell-cell transmission probably is the dominant route of virus propagation in chronically infected individuals. The cell-to-cell spread is more efficient than cell-free infection, which has been found in many other viruses including Vesicular Stomatitis Virus (VSV), Cytomegalovirus (CMV), Vaccinia Virus (VV), Human T-lymphotropic Virus (HTLV-I) and human immunodeficiency virus HIV (Sweet *et al*., 1999; Cole & Grose, 2003; Igakura *et al*., 2003; Simth & Law, 2004; Dale *et al*., 2011). To demonstrate the transmission routes of HCV infection, the McKeating's group developed a single-cycle co-culture assay to monitor single-cycle infection events, and to compare the efficiency of cell-free and cell-to-cell infection (Brimacombe *et al*., 2011; Meredith *et al*., 2013). They found that for all the strains tested, cell-cell transmission could be detected within 1h of co-culture of producer and target cells, whereas HCV cell-free infection was markedly delayed. More interestingly, they observed the difference in the ability to spread through cell-to-cell and cell-free routes for different HCV genotypes. In their study, HCV J6/JFH1 displayed 10-times higher rate of infection via cell-cell transmission compared to that of cell-free transmission, whereas HCV SA13/JFH1 and HK6A/JFH1 strains showed comparable rates of infection through both routes (Meredith *et al*., 2013).

Recently, it was found that exosomes from HCV-producing Huh7.5.1 cells can also transmit the infection to uninfected cells and establish a productive infection. Moreover, exosome-mediated HCV transmission was only partly inhibited by immunoglobulins purified from the serum of chronically infected patients. This new route of HCV transmission could offer a

potential mechanism for HCV immune evasion, and also an explanation for HCV re-infection that often occurs after liver transplantation (Ramakrishnaiah *et al.*, 2013).

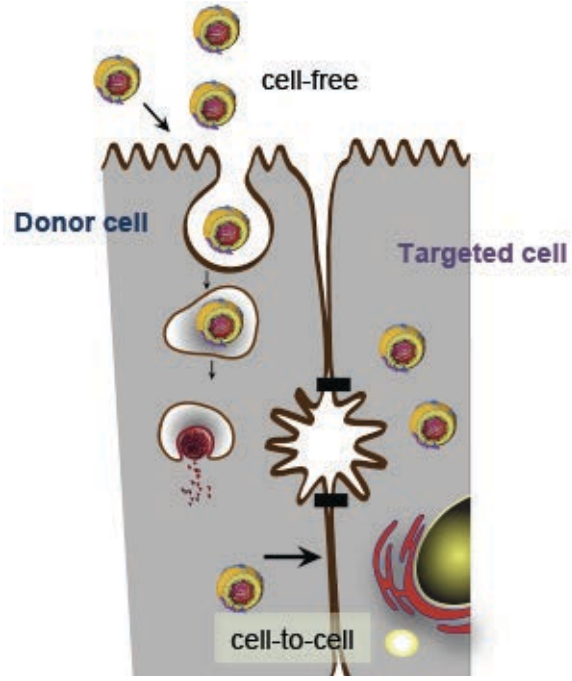


Fig.15: Schematic illustration of HCV cell-free and cell-cell transmission

Entry of cell-free enveloped virus is initiated by the interaction between virions and attachment factors, which concentrates virions on the cell surface and enables the lateral diffusion of virion and their subsequent interaction with specific co-receptors/entry mediators, thereby inducing conformational changes of viral particles. Some viruses enter cells through a pH-independent route by fusing their membranes with the plasma membrane of the cell surface, while other viruses such as HCV, penetrate into the cell by fusing their envelope with endosomal membranes after internalization of the virion via endocytosis. Although most viruses require a low pH environment to trigger the fusion with an endosomal membrane, some internalized viruses do not need such a pH change for fusion. These events ultimately lead to the uncoating of virions and release of viral genomes into the cytosol to initiate viral replication cycle (Sattentau *et al.*, 2008).

2. HCV entry into hepatocytes

Both HCV cell-free and cell-to-cell infections require at least four essential cellular entry factors, including scavenger receptor SRB1, tetraspanin CD81, tight junction molecules CLDN1 and OCLN (Pileri *et al.*,1998; Scarselli *et al.*, 2002; Evans *et al.*, 2007; Timpe *et al.*,

2008; Ploss *et al*., 2009; Brimacombe *et al*., 2011). Whether the exosome-mediated transmission also relies on these cellular factors remains unknown. HCV entry is a complex multiple-step event involving a number of cellular factors. Entry is initiated by the attachment of viral particles at the surface of host cells. Heparan sulfate proteoglycans (HSPGs) and low density lipoprotein receptor (LDLR) were indicated as the initial attachment factors interacting with HCV particles (Agnelle *et al*., 1999; Monazahian *et al*., 1999; Germi *et al*., 2002; Barth *et al*., 2003). (Fig.18 step1). After attachment, specific interactions between HCV envelope protein E2 and cellular factors SRB1 and CD81 are essential for the following steps of HCV entry involving other entry factors like the tight junction proteins CLDN1 and OCLN (Bartosch *et al*., 2003b; Stamataki *et al*., 2008; Harris *et al*., 2008; Harris *et al*., 2010). (Fig.18 step2-3). Eventually, HCV particles enter hepatocytes via clathrin-mediated endocytosis (Blanchard *et al*., 2006) (Fig.18 step4). Low pH environment in the early endosomes induces HCV fusion, leading to the uncoating of viral particle and delivering HCV genome RNA into the cytoplasm (Meertens *et al.*, 2006). (Fig.18 step5). Newly identified host factors such as epidermal growth factor receptor (EGFR), ephrin receptor A2 (Lupberger *et al*., 2011). Niemann-Pick C1-like 1 (NPC1L1) (Sainz *et al*., 2012) and transferrin receptor 1 (TfR1) (Martin *et al*., 2013) have also been described to be involved in the entry process of HCV. Furthermore, receptor tyrosine kinases (RTKs) like EGFR have been demonstrated to be involved in HCV entry by regulating CD81-CLDN1 co-receptor associations (Lupberger *et al*., 2011). The GTPase HRas was found to act as a critical host signal transducer required for the lateral membrane trafficking of CD81, which enables the assembly of tetraspanin receptor complexes (Zona *et al*., 2013).

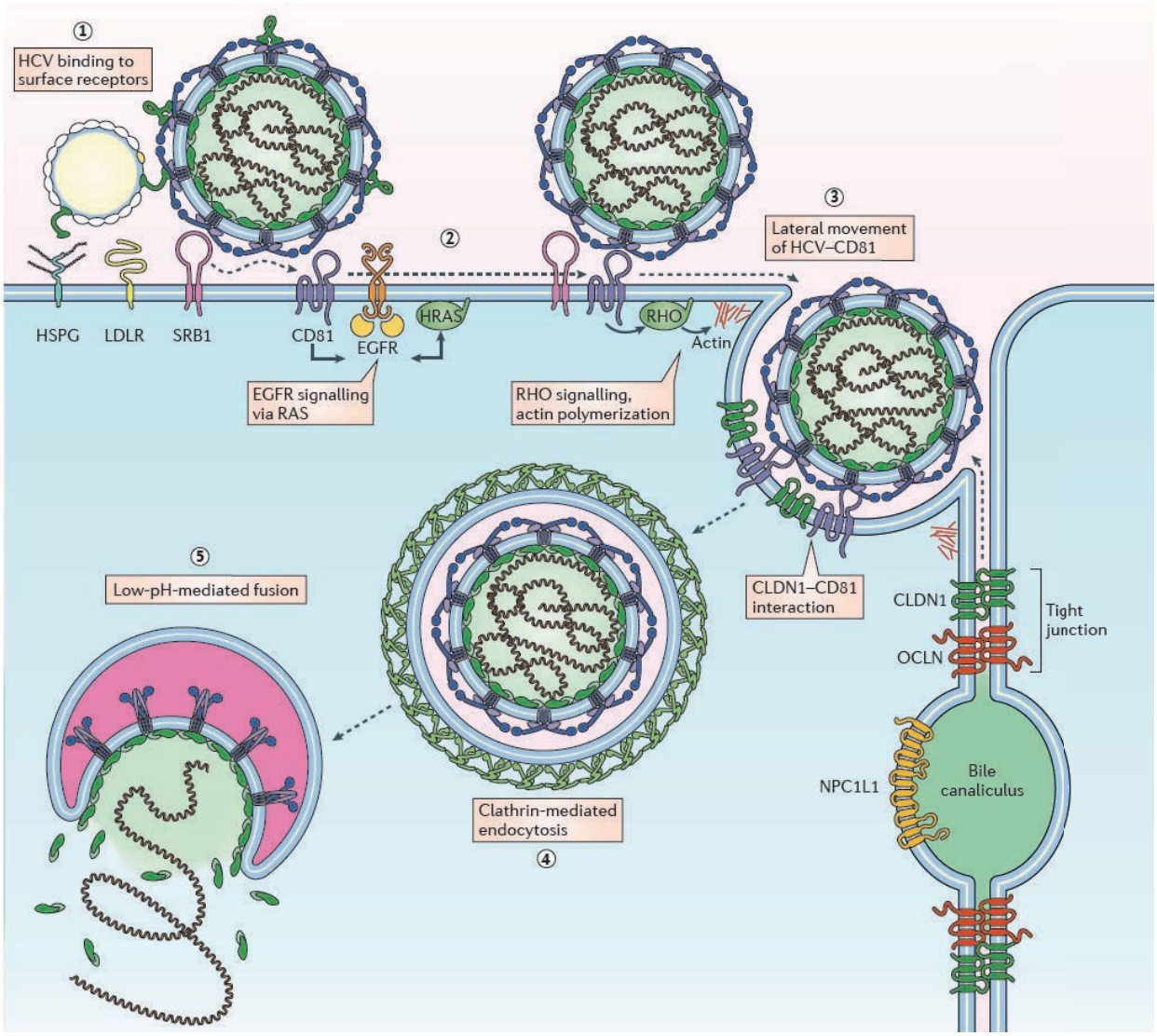


Fig. 16: Model of cell-free HCV entry into hepatocytes (Lindenbach & Rice, 2013).

HCV entry into hepatocytes depends on the direct interactions between viral particles and the molecules expressed on cell surface. However, entry can also be regulated indirectly by intracellular/extracellular proteins through interacting with these receptors /co-receptors. For instance, SRB1 partner protein PDZK1 has been demonstrated to positively affect the receptor SRB1 involvement in HCV entry by binding to the cytoplasmic carboxy-terminus of SRB1(Eyre *et al.*, 2010). By contrast, CD81 partner protein EWI-2wint, when ectopically expressed in hepatoma cells, was found to block HCV entry by inhibiting the interaction between HCV envelope proteins and CD81 (Rocha-Perugini *et al.*, 2008; Montpellier *et al.*, 2011). Like many viruses, HCV virus-receptor interactions not only determine the cellular tropism but also host range, which constitute the interspecies barriers for HCV infection. This was proven by the findings that expressing HCV-specific receptors CD81, SRB1, CLDN1

and OCLN in mouse hepatocytes rendered the murine cells permissive for HCV entry (Ploss *et al*., 2009). However, only CD81 and OCLN had to be of human origin. Also, this result demonstrated that CD81, SRB1, CLDN1 and OCLN are the minimum receptor complex required for HCV infection (Ploss *et al*., 2009). The establishment of stable viral replication in mouse hepatocytes which cannot be productively infected with HCV, further supported the conclusion that viral entry is a determinant for HCV host and tissue tropism (Zhu *et al*., 2003; Uprichard *et al*., 2006). Interestingly, a recent study also showed that adapting HCV to mouse CD81 can lead to viral entry into mouse cells in the absence of human HCV entry factors (Bitzegeio *et al*., 2010).

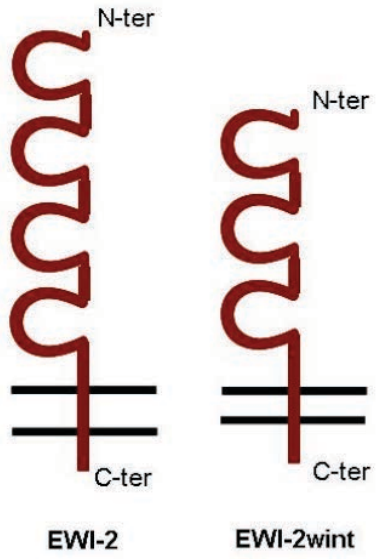


Fig. 17: Schematic structure of EWI-2 and EWI-2wint

EWI-2 is a major CD81 partner and a member of a novel Ig protein subfamily (Stipp *et al*., 2001). It is a component of the tetraspanin web in hepatocytes and lymphoid cells (Charrin *et al*., 2003). The horseshoes represents the immunoglobulin domains of EWI-2 and its cleavage product EWI-2wint, which is the EWI-2 without the N-terminal Ig-like domain (Stipp *et al*., 2001; Rocha-Perugini *et* al., 2008).

It is also noteworthy that there seems to exist a non-productive pathway for HCV entry, in which the interaction between the lipoprotein component of HCV particle and LDL receptor (LDLr) leads to a rapid internalization of virion without later interaction with other receptors at cell surface, then the internalized HCV virion probably undergoes degradation in internalized vesicles (Albecka *et al*., 2012). However, the LDLr-mediated HCV non-productive entry remains to be further defined.

3. HCV envelope glycoproteins in viral entry

HCV envelope glycoproteins E1E2 by being present on the surface of viral particle are the major players in viral entry, and also are the primary targets of anti-HCV neutralizing antibodies. Functional E1E2 form a non-covalent heterodimer (Fig.18a), which is essential for HCV infectivity (Michalak *et al*., 1997; Bartosch *et al*., 2003b; Hsu *et al*., 2003; Op De Beeck *et al*., 2004). E1 and E2 are known to mediate the interactions with HCV cellular receptors/co-receptors, and also the fusion between the viral envelope and cell membranes following HCV internalization. The role of E1 in the entry process of HCV is not very clear yet, though it has been proposed to assist the fusion process (Lavillet *et al*., 2007) and a recent study suggested its role as a modulator for HCV binding to receptors and membrane fusion (Douam *et al*., 2013). By contrast, a large body of studies have been focused on the glycoprotein E2. Using its soluble form, researchers identified the first (CD81) and the second co-receptors (SRB1) required for HCV infection (Pileri *et al*., 1998; Scarselli *et al*., 2002). The positions of functional domains in E2 which mediate receptor binding are shown in Fig.18b. There exist at least two CD81-binding sites located at discrete regions (amino acids 412-443 and 520-550) in E2 (Flint *et al*.,1999a). The hypervariable region 1 (HVR1) has been proposed to be responsible for the interaction of E2 with GAGs and SRB1 (Barth *et al*., 2003; Scarselli *et al*., 2002). Two regions in E2 (amino acids 416-430 and 600-620) have been mapped as contributing to the fusion process (Lavillet *et al*., 2007). In the past years, as no crystal structure of E2 was available, and based on the comparison to other Flaviviruses and the distribution of its disulfide bonds, E2 was predicted to be a class II fusion protein with three domains (Krey *et al*., 2010). Recently, the core of E2 protein was successfully crystallized, however, the x-ray and electron microscopy showed that E2 structure differs remarkably from the former predictions (Kong *et al*., 2013; Khan *et al*., 2014).

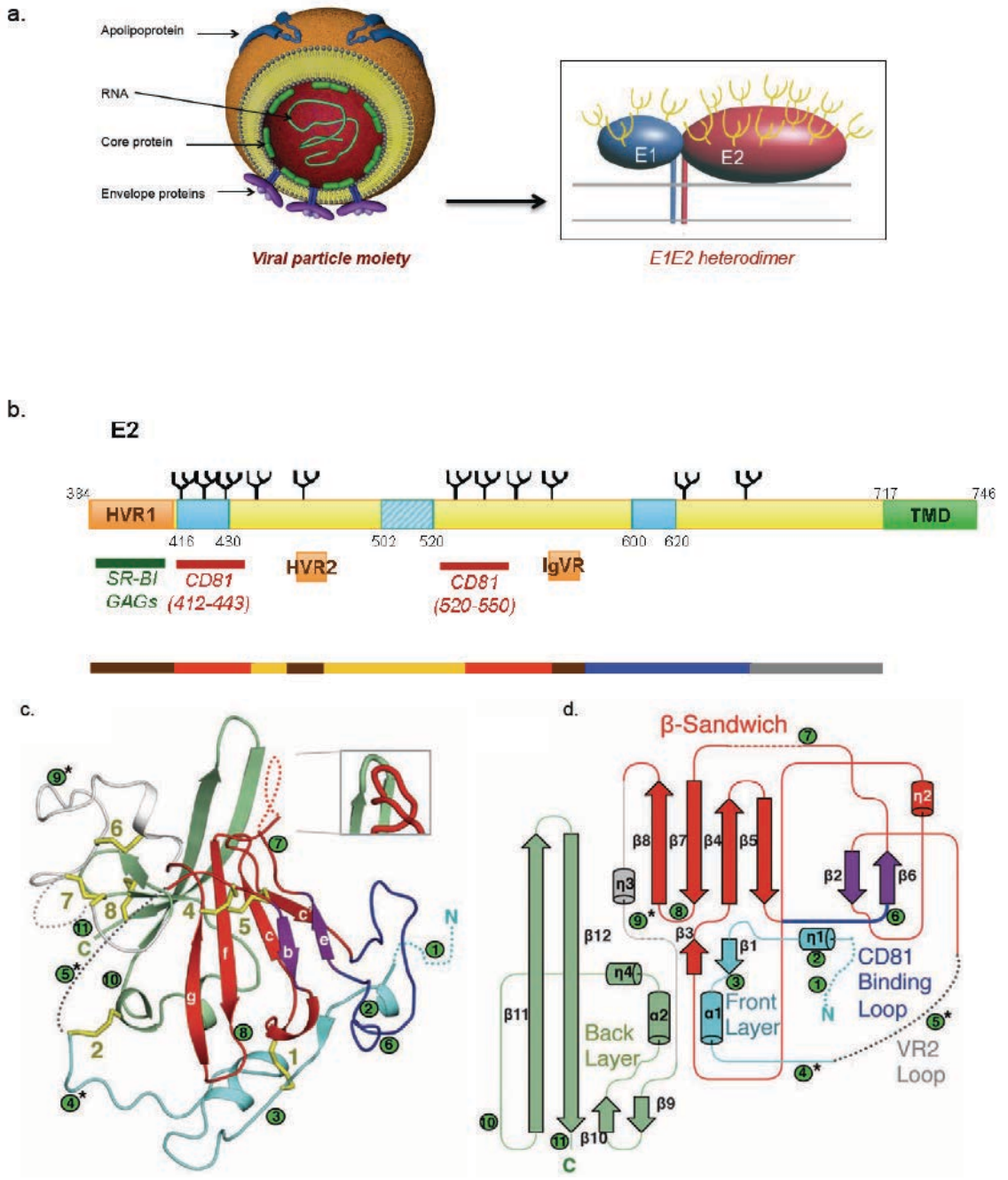


Fig. 18: Schematic illustration of HCV envelope proteins and functional domains of E2

a. HCV particle and topology of E1 and E2 envelope glycoproteins.
b. Organisation of E2 functional domains.
c. The crystal structure of HCV E2c is displayed as a cartoon representation and colored by structural components: N-terminal region (in cyan) forms the front layer; the outer (in purple) and inner (in red) sheets constitute the Ig b sandwich; the CD81 receptor binding loop is a bilobed structure (in blue) (aa519 to 535); a flexible region (in white) encompasses variable region 3 (VR3); and the back layer (aa597 to 645) is formed by two short helices, loops, and a four-stranded b sheet (in light green). Labeling of b-sandwich strands follows Ig-fold conventions. Disulfide bonds are shown as yellow sticks, N-linked glycans are indicated by green circles, both are numbered from the N-terminus. Asterisks indicate N-linked glycans deleted in the construct. Disordered regions in the structure are shown by dotted lines. The structure of a loop in the E2c structure from complex B that is disordered in complex A.
d. Cartoon representation of topology diagram of E2c following the same coloring scheme in c (Kong *et al.*, 2013).

Importantly, in addition to the identification of functional regions involved in HCV receptor recognition, some point mutations within or outside of the receptor-binding sites have been found to alter or modulate receptor binding. For instance, W529 or D535 of E2 residues were identified to be critical for CD81 binding, since the subsititution W529A or D535A completely abrogated HCV infectivity (Witteveldt *et al*., 2009). Mutation in JFH1 E2 at position 451 (G451R) has also been demonstrated to bind CD81 more efficiently, but displayed a reduced dependence on the receptor SRB1 (Grove *et al*., 2008). Also, introduction of N415D into JFH1 genome enhanced the binding of E2 to CD81, and other mutations including T416A, N417S, and I422L in the same region (aa 412-423) were similiar to JFH1 (N415D) in terms of receptor reactivity, suggesting the importance of this region in E2-mediated HCV entry (Dhillon *et al.*, 2010). Moreover, mutations in E1 (I374L) and E2 (I411V) of J6/JFH1 were observed to affect SRB1 usage exclusively in HCV cell-to-cell spread (Catanese *et al*., 2013a). In addition to the aa705-715 sequence in E2 stem region which has been identified to be essential for HCVcc entry (Albecka *et al*., 2011), single amino acids in the stem region were also found to influence the late step of HCV entry (Carlsen *et al*., 2013). Mutations in HCV envelope proteins can also affect HCV interactions with lipoproteins. For instance, I414T mutation increases HCV infectivity by altering the lipoprotein association with viral particle. Consequently, the buoyant density of viral particle was also altered (Tao *et al*., 2009). Another point mutation in E2 (G451R), resulting in 50% more cholesterol-contained in the mutant virus than the wild type, led to an enhanced NPL1C1-dependent cell entry of HCV particle (Sainz *et al*., 2012).

HCV envelope glycoproteins are highly glycosylated and the majority of the glycosylation sites on E1E2 are well conserved across all genotypes (Helle *et al*., 2007). Mutations of glycosylation sites at E1N1, E2N8 or E2N10 resulted in a decreased heterodimerization of recombinant E1E2 and inefficient incorporation of envelope proteins into HCVpp (Goffard *et al*., 2005). Interestingly, other glycans (E2N1, E2N2, E2N4 and E2N6) also modulate the accessibility of the CD81 binding site (Falkowska *et al*., 2007; Helle *et al*., 2010). Furthermore, adaptive muations which do not harbor the E2N6 glycan have emerged in cell culture, suggesting that enhanced interaction with the receptor CD81 contributes to the better fitness of these viral mutants *in vitro* (Bunygoku *et al*., 2009; Delgrange *et al*., 2007).

4. Hypervariable region 1 of envelope glycoprotein E2

HVR1 is a 27 amino acids segment located at the N-terminal of E2 (Fig.18b) (Hijikata *et al*., 1991; Ogata *et al*., 1991; Weiner *et al*., 1991). It is the most variable sequence among HCV isolates, however the variability of HVR1 is not random as a conservation of physico-chemical properties of some amino acids and the positive charges of others has been observed in this region (Penin *et al*., 2001; Callens *et al*., 2005). Due to its high variability and strong immunogenicity, HVR1 was thought to function as an immunological decoy, which can stimulate a strong immune response responsible for the variant selection but ineffective to clear HCV from the body (Ray *et al*., 1991). It was found that amino acid substitutions occurred almost exclusively within HVR1 during HCV acute infection (Farci *et al*., 2000). Also, in an evolutionary study of HVR1 from chronically infected, IFN-treated patients, the evolution of HVR1 region was shown to be driven by a selective pressure independent of the complexity or diversity of the viral population before treatment (Alfonso *et al*., 2004). Also, HVR1 variability was found to be dramatically reduced in hypogammaglobulinemia patients (Booth *et al*., 1998). Together, these findings confirm that HVR1 is a critical target of the selective pressure of the host immune system, and it might play a role in the progression to chronic HCV infection (Weiner *et al*., 1992; Kato *et al*., 1993; Pawlotsky *et al*.,1999). Moreover, HVR1 genetic complexity has been suggested to be an independent predictor for the response to IFN-alpha treatment in HCV chronically infected patients (Pawlotsky *et al*., 1998; Grahovac *et al*., 2000). It is also important to note that HCV infection in chimpanzees can be prevented by pretreatment with a hyperimmune serum directed against HVR1 (Farci *et al*., 1996; Farci *et al*., 2006). Furthermore, in acute HCV infected patients, early antibody response against HVR1 has been shown to be associated with virus clearance (Allander *et al*., 1997). These observation indicated that HVR1 contains at least one neutralization epitope; however, this region has also been demonstrated to shield important conserved neutralizing epitopes, impairing virus neutralization of all major genotypes in the HCVcc system (Prentoe *et al*., 2011).

In chimpanzees, HCV lacking HVR1 is still infectious, though its infectivity is significantly reduced. However, this attenuation can be compensated by individual mutations within envelope proteins, indicating that though HVR1 deletion decreases HCV replication *in vivo*, it is not critical for HCV viability (Forns *et al*., 2000). An *in vitro* study showed that HVR1 deletion differentially affected the viability of HCV recombinant viruses from genotype 1 to

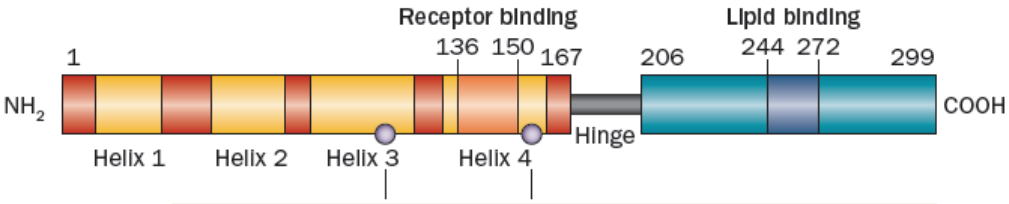
6. HCV 2a and its HVR1-deleted viruses exhibited similar infectivity, and the viability of 2a, 5a, and 6a genotypes lacking HVR1 viruses did not require cell-culture adaptive mutations. For genotypes 1a, 1b, 2b and 3a, the appearance of adaptive mutations can rescue the impaired infectivity of corresponding HVR1-deleted viruses. While for all genotypes, HVR1 deletion led to increased density and decreased heterogeneity of infectious particles, with the density of infectious particle shifting from a range of 1.0-1.1g/ml to a single peak at 1.1g/ml. This observation indicated that the infectivity of low-density viral population depends on HVR1 (Prentoe *et al*., 2011). It is worthy to note that HVR1 deletion has no influence on the glycosylation and incorporation of envelope proteins E1 and E2, which are important for virus assembly and entry (Bankwitz *et al*., 2010; Prentoe *et al*., 2014). HVR1 was demonstrated to directly participate in the binding of HCV E2 to SRB1, mediating direct E2-SRB1 interaction (Scarselli *et al*., 2002; Bartosch *et al*., 2003b). HVR1-deleted HCVpp or HCVcc cannot be inhibited by anti-SRB1 or SRB1-specific immunoglobulins, indicating their loss on SRB1 dependence (Bartosch *et al*., 2003b; Bankwitz *et al*., 2010). Comparing to its native form, recombinant E2 lacking HVR1 showed decreased binding to SRB1, but improved binding to CD81 (Scarselli *et al*., 2002; Roccasecca *et al*., 2003). However, in the context of HCVcc, both wild-type and HVR1-deleted viruses were comparably neutralized by anti-CD81 specific antibodies, suggesting that HVR1 deletion did not affect CD81 receptor usage (Bankwitz *et al*., 2010). This conclusion was further confirmed by comparing virus infection of several HCV isolates and their HVR1-deleted mutants in CD81 specifically silenced Huh7.5 cell (Prentoe *et al*., 2014). So far, it is considered that HVR1 of E2 probably functions in HCV entry, involving the usage the receptor SRB1 in a virion-associated apoE independent way.

5. HCV particle component apoE

As discussed above apolipoprotein apoE is incorporated into HCV viral particle. Being exposed on the surface of virion, apoE has been show to play an important role in HCV entry through interacting with cellular factors including Heparan Sulfate Proteoglycans (HSPGs), LDLr and SRB1 (Germi *et al*., 2002; Barth *et al*., 2003; Dao Thi *et al*., 2012). Besides the important role of apoE in HCV infection, apoE has also been reported to be involved in other serious human diseases including Alzheimer's Disease (AD) and Herpes Simplex Virus (HSV) infection.

5.1 ApoE protein and its isoforms in lipid metabolism and in AD

ApoE is a member of soluble apolipoprotein family, composed of 299 amino acids with a molecular weight of approximately 34kDa (Zannis & Breslow *et al*., 1982; Mahley & Rall , 2000). Human apoE gene locates on chromosome 19 in a cluster with apoCI/CII gene complex. It encodes three protein isoforms: apo-E2, -E3 and -E4, differing from each other by one or two amino acids at the position 112 and 158 (Myklebost *et al*., 1984; Scott *et al*.,1985; Eichner *et al*., 2002). As shown in Fig.19, for apo-E3, the residue 112 and 158 are a Cysteine and Arginine, respectively, while apo-E2 has Cysteine residues and apo-E4 has Arginine residues at both positions (Weisgraber *et al*., 1981; Zannis *et al*., 1982). The amino acid differences make apoE exhibit isoform-specific receptor binding and play different roles in apoE-involved diseases. Notably, ApoE allele frequencies vary in different populations worldwide. It has been found that apo-E3 is present in up to 77.9% individuals, whereas apo-E2 has the lowest frequence at 8.4%, apo-E4 occurs in 13.7% individuals (Farrer *et al*., 1997). The polymorphism of apoE allele results in six different phenotypes including three homozygous phenotypes (apoE4/4, apoE3/3, apoE2/2), and three heterozygous phenotypes (apoE4/3, apoE3/2 and apoE4/2) (Zannis *et al*., 1982).



	Isoform-specific amino acid difference		Allele frequency (%)	
	112	158	General	AD
Apo-E2	Cys	Cys	8.4	3.9
Apo-E3	Cys	Arg	77.9	59.4
Apo-E4	Arg	Arg	13.7	36.7

Fig.19: Diagrammatic sketch of ApoE isoforms and allele frequencies in AD (Liu *et al*., 2013)

ApoE was initially shown to be involved in the lipid (cholesterol and triglyceride) metabolism and cardiovascular disorders (Eichner *et al*., 1993; Lehtinen *et al*.,1995; Wang et al.,1995). It is known as a major component of VLDL, HDL and chylomicron (CM) remnant, and plays multiple roles in the hepatic and extrahepatic lipid and lipoprotein metabolism. ApoE acts as a cofactor in the hepatic VLDL biogenesis and release, participates in the hydrolysis of VLDL

remnants into LDL and mediates the cellular uptake of lipoprotein remnants by binding to cell surface HSPGs as well as members of the LDL receptor (LDLr) family, which includes LDLr, LDL receptor-related protein (LRP), VLDL receptor and apoE receptor 2 (Jofre-Monseny *et al*., 2008; Holtzman *et al*., 2012). Plasma apoE primarily originates from liver VLDL synthesis, though extra-hepatic tissues such as kidney, adrenal glands, b<rain glial cells and tissue macrophages together are estimated to produce 20-40% of total apoE (Blue *et al*., 1983; Minhane *et al*., 2007). Importantly, plasma apoE level is a significant determinant of triglyceride-rich lipoprotein metabolism. As an independent factor, plasma apoE concentration alone can explain 20-40% variability of blood triglyceride levels (Salah *et al*., 1997; Hagberg *et al*., 2000; Haddy *et al*., 2002). Overexpression and accumulation of apoE was found to stimulate hepatic VLDL triglyceride and impair VLDL lipolysis, thus resulting in a lipid metabolic disorder with the feature of high plasma triglyceride level, called hypertriglyceridemia (Huang *et al*., 1998). Conversely, very low plasma apoE level can impair the metabolism of triglyceride-rich lipoprotein and the clearance of lipoprotein remnants from the plasma. Hence, appropriate plasma apoE level is considerably important for the triglyceride and cholesterol homeostasis.

It is noteworthy that apoE is also the major apolipoprotein existing in the cerebrospinal fluid (CSF), which is largely produced by astrocytes and in charge of the transport of cholesterol and phospholipid in the brain. Indeed, apoE and cholesterol are so crucial for the fundamental activities of central nervous system (CNS) that the brain can independently regulate their synthesis. Plasma apoE and cholestrol do not seem to exchange with the CNS apoE and cholestrol through the blood-brain barrier (BBB) (Linton *et al*.,1999). In the brain, the availability of cholesterol and aging (brain cholesterol synthesis decreases with age) play critical roles in the synaptic development of neurons and neuronal plasticity (de Chaves *et al*., 1997; Thiele *et al*., 2000; de Chaves *et al*., 2001; Koudinov & Koudinova, 2001; Mauch *et al*., 2001; Koudinov & Koudinova, 2002; Goritz *et al*., 2005). The role of ApoE in synaptic functions mainly involves cholesterol transport, though several other mechanisms have also been proposed (de Chaves & Narayanaswami, 2008). Cholesterol is the essential constituent of cell membranes, and of the myelin sheaths which wrap the axons of neurons (Saher *et al*., 2005). Cholesterol reduction was found to impair the exocytosis of synaptic vesicles, implicating the important role of cholesterol in the release of neurotransmitter (Linetti *et al*., 2010). Therefore, apoE is very pivotal in maintaining the normal brain function. The effect of

apoE on synaptic plasticity also appears isoform-specific (Castellano *et al*., 2011). For instance, genetic expression of apoE4 in mice significantly reduces the excitatory synaptic transmission, comparing to mice expressing apoE3 (Wang *et al*., 2005). Also, apoE4 mice were found to have less synapses and dendritic spines per neuron (Cambon *et al*., 2000; Ji *et al*., 2003). Moreover, apoE4 mice were shown to be more sensitive to the cerebral ischemia (Horsburgh *et al*., 2000), and less capable in recovering from brain injury than apoE3 mice (Sabo *et al*., 2000). Increasing studies have demonstrated that apoE4 is the key genetic determinant of AD which is characteristic of abundant amyloid-β plaques aggregating in the cortex, and other neurodegenerative diseases in the brain (Corder *et al*., 1993; Mahley *et al*., 2006; Chen *et al*., 2010).

Table 1. ApoE isoforms and their functional difference and associated disorders

Isoform	LDL receptor binding	Lipoprotein preference	Associated disorders
E2	Low	HDL	Type III hyperlipoproteinemia
E3	High	HDL	Unknown
E4	High	VLDL,CM	AD and other neurological conditions;atherosclerosis

Importantly, apoE4 was also found to be less efficient in amyloid β (Aβ) peptides clearance than apoE3 in mouse models expressing human apoE isoforms (Castellano *et al*., 2011). The possible mechanism is that apoE4-containing lipoproteins bind Aβ with lower affinity than apoE3-containing lipoproteins (LaDu *et al*., 1994; LaDu *et al*., 1995), therefore apoE4-lipoprotein particles are defective in sequestering Aβ and promoting cellular uptake and degradation of apoE-Aβ complexes (Kim *et al*., 2009). Furthermore, apoE4 also contributes to AD pathogenesis through Aβ-independent mechanisms, involving synaptic plasticity, CNS cholesterol homeostasis, neurogenesis and neuroinflammation (Liu *et al*., 2013).

The structural and biophysical differences among apoE isoforms can provide the insights on their different effects on different apoE-related diseases. It is well documented that apoE contains two functional domains separated by a hinge region (Fig.19). The 136-150 region in the N-terminal domain was proved to be the site for receptor binding, and the residues 244-272 region in C-terminal domain is responsible for the lipid-binding (Wetterau *et al*., 1998; Morrow *et al*., 2000). Single amino acid differences Cys/Arg at the position 112 and 158

make the receptor-binding function of apoE exhibit isoform-specificity. Studies showed that both apoE3 and apoE4 can efficiently compete with ^{125}I-LDL for binding to the cell surface, while apoE2 was apparently ineffective in binding. It was considered that a positive charge at aa position 158 is critical for the normal receptor binding activity (Weisgraber *et al*., 1982). Thus, the primary structure of apoE seems to determinate its receptor-binding function. The structure of the N-terminal domain (aa1-167) of apoE has been solved by X-ray crystallography. It appears as a helix bundle and contains four helices, with the LDL receptor-binding site located in the Helix-4 (Fig.20). Interestingly, the conformational stability of the N-terminal domain differs significantly among the three apoE isoforms (Morrow *et al*., 2000; Acharya *et al*., 2002). Moreover, the conformation of apoE shows a great heterogeneity when apoE binds to lipids (Krul *et al*., 1988), varying in size and shape. Importantly, lipid-bound apoE shows higher binding affinity for LDL receptors than lipid-free apoE (Funahashi *et al*., 1989) (Fig.20). And the receptor binding activity of lipid-bound apoE depends on the total size, lipid composition and also the existence of other apolipoproteins in the apoE-containing remnants (Kowal *et al*., 1980; Gianturco *et al*., 1983; Ishikawa *et al*., 1988; Weisgraber *et al*., 1990). With regard to the C-terminal domain of apoE, it was shown to be crucial for the apoE-mediated plasma cholesterol clearance. Interestingly, apoE4 was found to bind to VLDL better than apoE3 (Kitajima *et al*., 2006; Sakamoto *et al*., 2008; Nguyen *et al*., 2010). However, the enhanced VLDL-binding of apoE4 led to an impaired lipolysis of VLDL in apoE4-expressing mice (Li *et al*., 2013). These data fit with the observation that apoE4 is associated with an increased risk of developping coronary artery disease (Davignon *et al*., 1988).

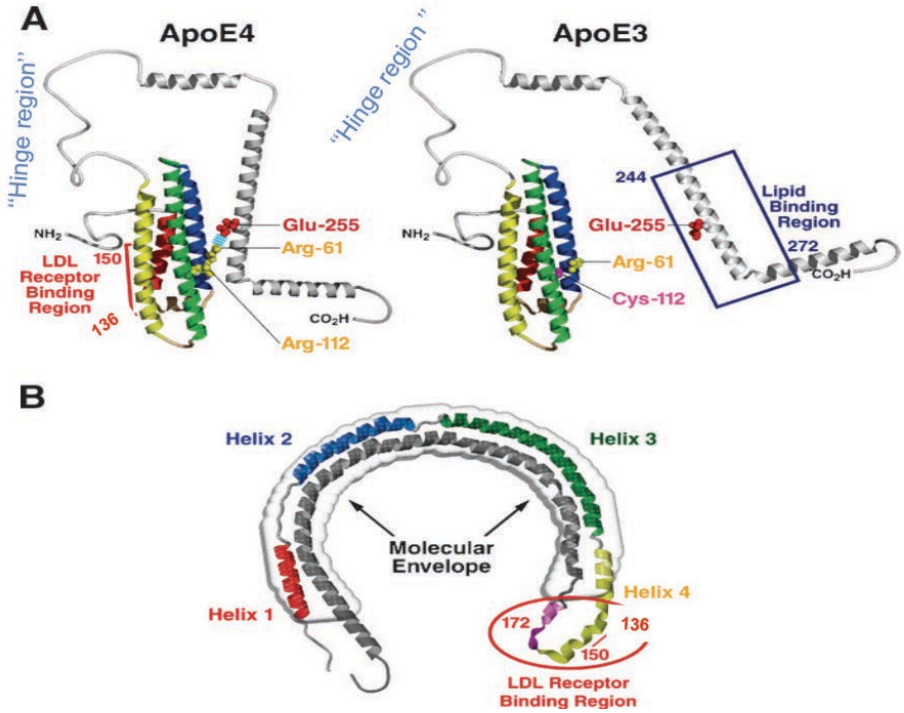


Fig. 20: Model of the structure of lipid-free apoE (A) and lipid-bound apoE (B) (Mahley & Huang, 2009)

Notably, in addition to the important role of apoE in clearing plasma cholesterol, the relative difference between apoE3 and apoE4 in VLDL-binding preference has a significant influence on the distribution of plasma cholesterol between VLDL and HDL factions. The VLDL-cholesterol/HDL-cholesterol ratio was found to be higher in the apoE4 expressing mice, compared to the mice expressing a similar level of apoE3 (Li *et al*., 2013). After synthesis in the liver and secretion into the plasma, VLDL encounters lipoprotein lipase (LPL) in the circulation and undergoes LPL-mediated hydrolysis, with the requirement of apoC-II as a cofactor (Kinnunen *et al*., 1977), leading to the production of intermediate density lipoprotein (IDL) and progressively smaller lipoprotein remnants (Li *et al*., 2013)(Fig.21, represented by circles in purple). The surface components and apoE of these particles interact with LDLr, mediating their clearance from the plasma. The improved VLDL-binding activity of apoE4 results in higher concentration of apoE4 on the VLDL surface, thus more apoC-II is displaced from the VLDL particles and the TG hydrolysis catalyzed by LPL is inhibited (Weisgarber *et al*., 1990).

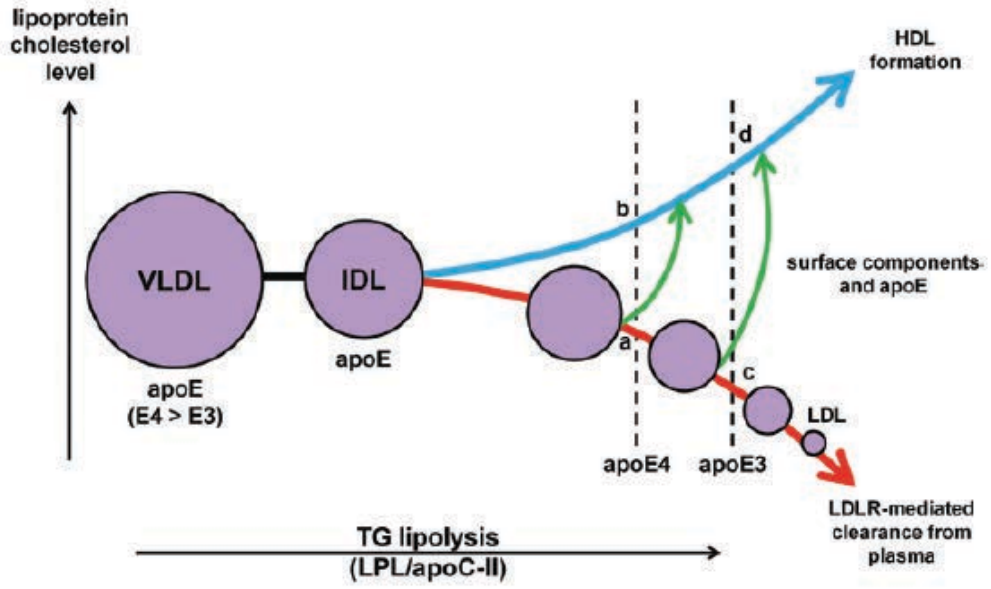


Fig. 21: Shematic comparing the influence of apoE3 and apoE4 on plasma VLDL particle catabolism.

After VLDL synthesis and secretion from the liver, the triglycerides (TG) in VLDL particles are hydrolyzed in the bloodstream by lipoprotein lipase (LPL) with apoCII functioning as a cofactor, which leads to the formation of intermediate-density lipoprotein (IDL). Then, IDL particles are progressively catabolized into small low-density lipoprotein (LDL) remnant particles, with the removal of core TG and excess surface components (including cholesterol, phospholipid and apoE) being released into the high-density lipoprotein (HDL) pool (the upper curved arrow shows the HDL-cholesterol level, the lower curved arrow represents the cholesterol level in the VLDL/IDL pool). ApoE on the surface of these remnant particles mediates their interactions with LDL receptor, promoting their clearance from the plasma. When apoE isoform apoE3 is expressed, apoE3 partitions evenly between the VLDL and HDL pools. The VLDL clearance and the HDL formation is optimal. Points c and d respectively represent the VLDL/IDL-cholesterol and the HDL-cholesterol levels in the plasma. While in the case of apoE4 being expressed, due to its higher lipid affinity (compared to apoE3), more apoE4 binds on the surface of VLDL, leading to an enhanced displacement of apoC-II from VLDL particles. Consequently, LPL-mediated TG lipolysis is relatively limitated. Thus, at the same expressing level, apoE4 expression correlates with a relative limitation of the lipolysis cascade. Complete formation of lipid remants are impaired. Points a/b represents the ratio of apoE4 VLDL Cholesterol/HDL-cholesterol, which is higher than that of apoE3 (points c/d) (Li *et al*., 2013).

5.2 ApoE in infectious diseases

Apart from the important roles of apoE in lipoprotein metabolism, Alzheimer's disease and cardiovascular disorders (Mahley *et al*., 1998; Mahley & Ji, 1999; Mahley & Rall, 2000; Eichner *et al*., 2002), apoE genotype was also shown to have a considerable influence on some viral diseases such as those caused by HIV, HSV and HCV. In a large scale genetic-epidemiologic study, apoE 4/4 genotype was discovered to be associated with an accelerated

HIV disease course in HIV^+ patients, compared to apoE3/3 genotype (Burt *et al*., 2008). *In vitro* study showed that HIV fusion/cell entry was enhanced in the presence of recombinant apoE4 but not apoE3. Several mechanisms have been proposed to explain this observation (Fig.22). For instance, apoE3 and apoE4 have comparable affinity for receptor binding, but their capacities of lipid-binding are very different, which could result in differential abilities of apoE3 and apoE4 to bind to the cholesterol component of HIV envelope or the cell surface cholesterol and phospholipid-rich rafts, both were found to be essential for HIV entry and virus assembly (Manes *et al*., 2000; Campbell *et al*., 2001, Liao *et al*., 2003).

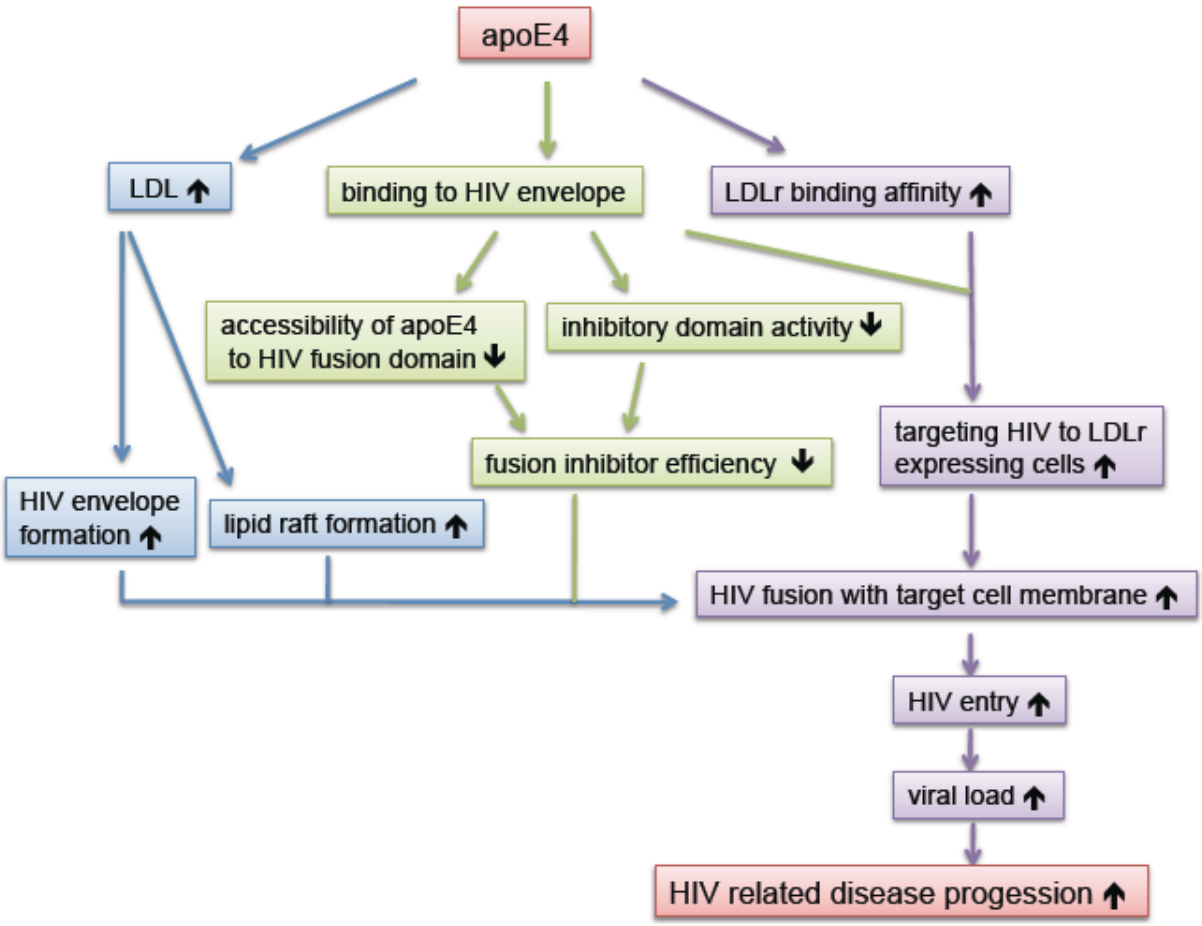


Fig. 22: Potential mechanisms for apoE4-mediated accelerated progression of HIV diseases Adapted from (Kuhlmann *et al*., 2010).

HSV is an enveloped double-stranded DNA virus, including two types. HSV-1 usually can cause labial cutaneous lesion and it can also induce a neurotropic infectious disease, while HSV-2 is responsible for genital infections. Similar to HIV infection, apoE genotypes did not

show any influence on the susceptibility to HSV-1 infection (Beffert *et al*., 1998; Burt *et al*., 2008). However, it has been found that apoE4 apparently induced HSV-1 immediate early gene (IEG) expression in neurons and the establishment of HSV-1 latency (Miller & Federoff, 2008). Furthermore, data from a corhort study showed that apoE4 allele frequency was much higher in HSV-1 positive AD patients compared to HSV-1 negative- AD and non-AD groups. HSV-1 infection alone was not sufficient for the development of AD, but when it combines with apoE4 allele carriage, the risk of AD was dramatically increased (Itzhaki *et al*., 1997). Importantly, apoE status was found to determine HSV-1 transmission in a gender-specific manner (Burgos *et al*., 2007).

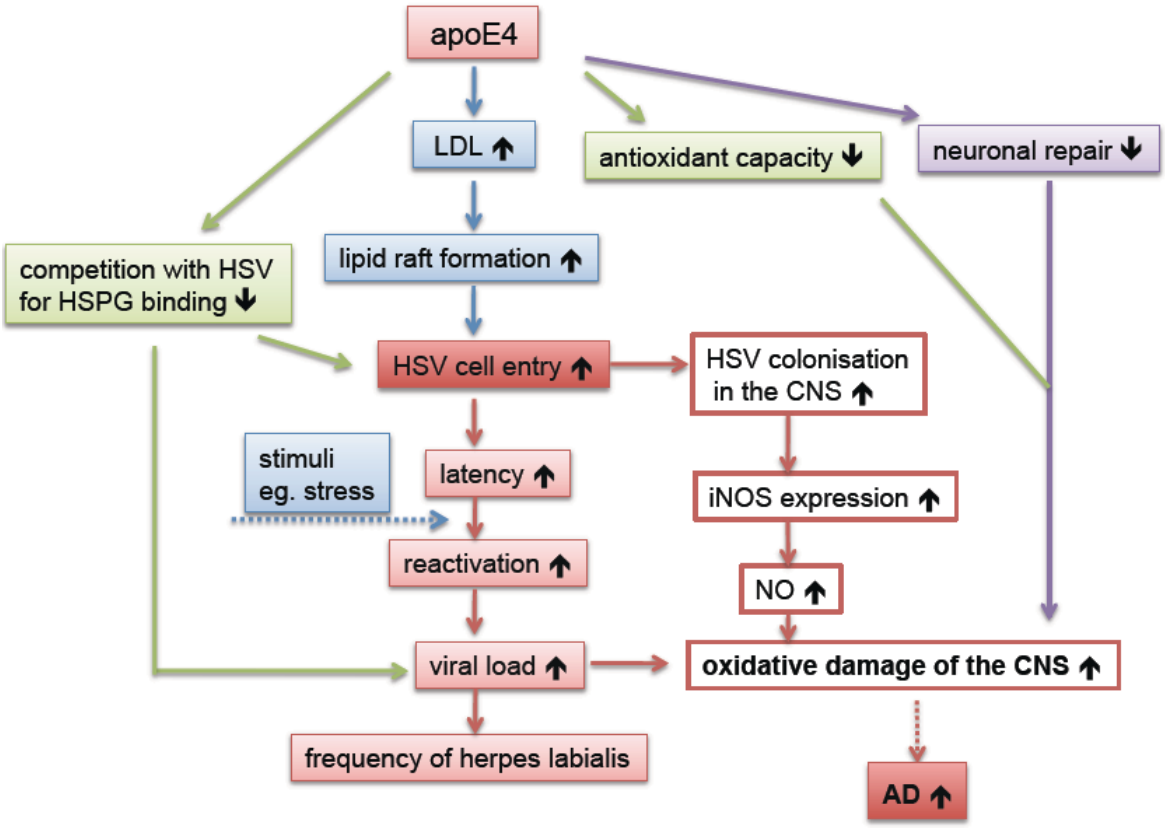


Fig. 23: Possible mechanisms to explain the effects of apoE4 on the outcome of HSV-1 infection and the increased susceptibility to AD. Adapted from (Kuhmann *et al*., 2010).

Interestingly, it has also been found that mice expressing human apoE4 had very high level of virus in the brain, compared to mice expressing human apoE3. However, no significant differences of virus level in the blood or in peripheral organs were found between these apoE3 and apoE4 transgenic mice, suggesting that apoE genotypes also affect the entry of HSV-1 into the CNS (Burgos *et al*., 2002; Burgos *et al*., 2003). HSV-1 invasion of the brain is more efficient in the context of apoE4 protein expression compared to apoE3 protein expression, providing a novel potential mechanism for the increased susceptibility of

developing AD in HSV-1 infected patients. Also, latent HSV-1 level in the CNS was found to be much higher in apoE4 expressing mouse compared to apoE3 mouse (Burgos *et al*., 2006), indicating the HSV-1 colonisation in the CNS where frequent virus activation leads to an increased oxidative damage in the brain. Briefly, several possible mechanisms may explain the significantly increased risk of AD in the combination of the apoE4 allele carriage and HSV-1 infection (Fig.23).

Table 2. Effects of apoE genotype on HCV infection and the outcome

Subjects and Profile	Parameter	Outcome	Reference
156 HCV patients: 111 chronically infected and 45 with cleared infection,and 104 non-HCV infected patients	-risk of HCV infection -risk of severe liver disease caused by chronic HCV infection -risk of non-HCV associated liver disease and degree of disease	ε2=ε3=ε4 non-ε4>ε4 ε2=ε3=ε4	Wozniak MA *et al*. 2002 Hepatol.
506 chronically infected HCV patients	response to antiviral treatment of HCV infection	non-ε4>ε4	Muller T *et al*.2003 Hepatol.
420 Caucasian HCV patients:312 chronically infected and 108 with cleared infection,and288 healthy controls	risk of chronic HCV infection	ε3>ε4>ε2	Price DA *et al*.2006 Gut
701 HCV patients chronically infected,523healthy controls, and 283 patients with non-HCV associated liver diseases	-risk of chronic HCV infection -risk of severe non-HCV associated liver disease	non-ε4>ε4 ε4=non-ε4	Muller T *et al*.2007 Z Gastroenterol

ϵ : apoE allele (Price *et al*., 2006).

By contrast, apoE polymorphism was found to have no influence on the risk of acquiring HCV infection (Wozniak *et al*., 2002). However apoE isoforms differentially affect the outcome of HCV infection and the course of HCV-related liver diseases (Table. 2). It has been reported that apoE3 allele is strongly associated with persistent HCV infection (Table.3), while genotypes carrying apoE2 allele (E2,E3 and E2,E4) are linked to a reduced risk of progressing into HCV chronic infection (Price *et al*., 2006). Surprisingly, apoE4 which acts as an adverse factor in diseases related to HIV and HSV-1 infection, was shown to protect against severe liver disease caused by HCV infection, but not against liver damage induced by non-HCV factors (Wozniak *et al*., 2002). ApoE is a major determinant of plasma lipoprotein metabolism, affecting plasma lipid level in an isoform-specific manner. ApoE4 allele has been found to be associated with elevated LDL level in the blood (Davignon *et al*., 1988). Thus, it has been suggested that increased LDL concentrations lead to a stronger competition with HCV particles for the LDLr-binding at the surface of hepatocytes (Fig.24). Moreover, LDLr expression is inversely modulated by the concentration of plasma LDL (Davignon *et*

al., 1988). Eventually, apoE4 genotype may prevent liver damage through sharply reducing HCV infection. Recently, apoE has also been demonstrated to be associated with the sensitivity to IFN therapy in HCV genotype 1 infected patients (Sheridan *et al*., 2010). However, the influence of apoE polymorphism on the outcome of anti-HCV treatment has not been reported (Table. 2).

Table 3. ApoE genotype and allele distribution (in %) in HCV-infected patients and healthy controls

ApoE genotype	Controls(n=288)	HCV cohort (n=420)	p Value
E2,E2	5(2%)*	0*	0.0067
E2,E3	32(11%)	39(9%)	ns
E2,E4	11(4%)	9(2%)	ns
E3,E3	167(58%)	263(63%)	ns
E3,E4	66(23%)	105(25%)	ns
E4,E4	7(2%)	4(1%)	ns

ns: no significant difference (Price *et al*., 2006).

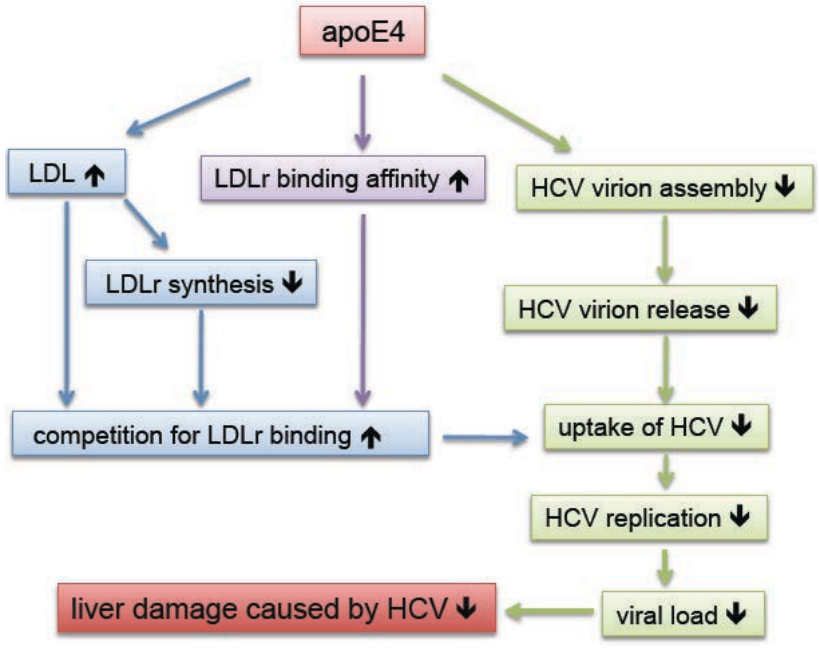


Fig. 24: Possible mechanisms for apoE4 protecting severe liver damage caused by HCV
Adapted from (Kuhlmann *et al*., 2010).

5.3 Influence of apoE isoforms in the HCV life cycle

The impact of apoE isoforms on HCV infection and the role of apoE protein in the HCV life cycle have been well documented in cell culture. In the study of Hishiki *et al.*, plasmids expressing apoE isoforms (apoE2, apoE3, and apoE4) were separately transfected into Huh7 cells in which the endogenous expression of apoE was inhibited. Infecting these cells with HCV led to a comparable production of infectious HCV particles in Huh7 cells expressing exogenous apoE3 or apoE4, while Huh7 cells expressing ectopic apoE2 released substantially less infectious virus. Also, the infectivity of HCV generated from apoE2 expressing cells was significantly decreased, comparing to that of HCV bearing apoE3 or apoE4 (HCV/apoE3 or HCV/apoE4). However, these observations were not confirmed by other groups (Da Costa *et al*., 2012; Long *et al*., 2011). Furthemore, Cun *et al* demonstrated that the C-terminal α-helix of apoE is required for interaction with NS5A and assembly of infectious viral particles. Three apoE isoforms did not show significant difference in the apoE-NS5A interaction, suggesting apoE isoforms do not effect HCV assembly and infectivity (Cun *et al*.,2010).

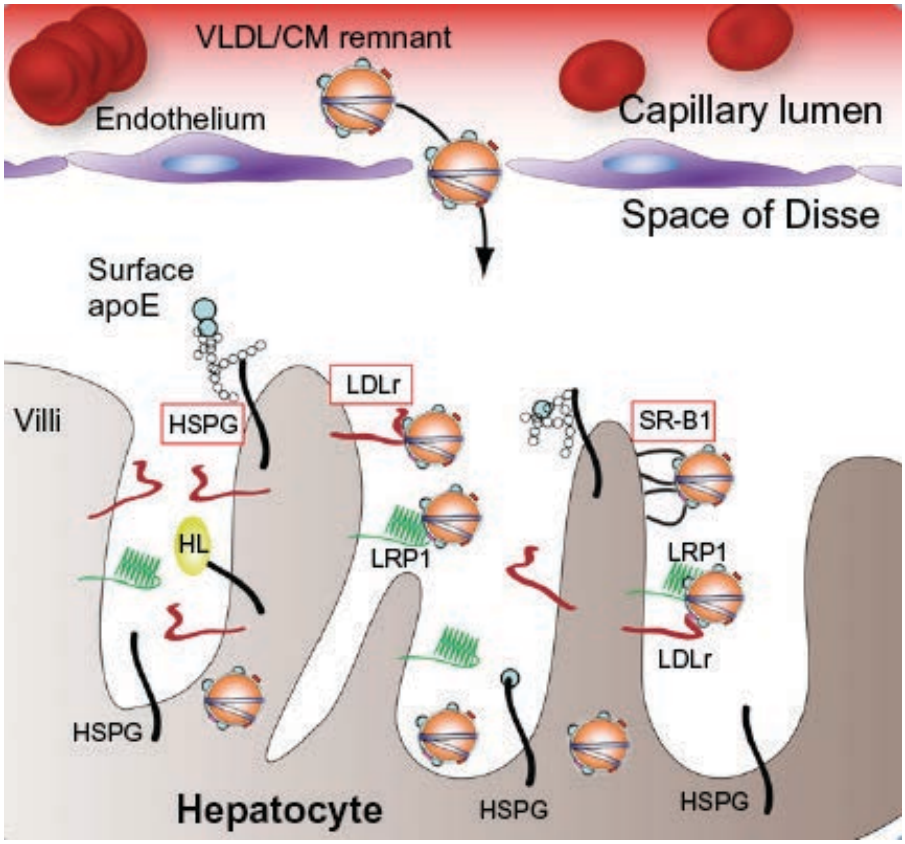


Fig.25: HepaticTriglyceride-rich lipoprotein remnant clearance (Bassendie *et al*., 2011).

The remnant particles are sequestrated into the space of Disse through the fenestrae of the endothelium. Surface apoE on the remnant particle binds to its receptors including two endocytic receptors (HSPGs and LDLr) and one docking receptor SRB1, mediating the normal hepatic remnant metabolism (Williams & Chen, 2010). All these receptors are involved in HCV entry.

In addition to the LDLr-binding, the HCV viral component apoE also binds to HSPGs, mediating the attachment of HCV particles at host cell surface to initiate viral infection. While, under the physiological condition, by being present on the surface of lipoprotein remnant particles, apoE serves as a high-affinity ligand for binding to the LDL receptor family and HSPGs and therefore plays a critical role in the hepatic uptake and clearance of plasma remnant lipoproteins (Mahley & Huang, 2007). It was proposed that apoE also has a function in the hepatic VLDL assembly and/or secretion, in addition to its role in the VLDL remnant clearance.

ApoE was found to be present in nascent VLDL particle in the Golgi compartment of rat hepatocytes (Hamilton *et al*., 1991). However, it is not clear whether the apoE molecule found in the secreted VLDL is due to the integration during the process of hepatic VLDL synthesis. Unlike apoB, apoE is not the structural component of VLDL and it exchanges between VLDL and HDL particles (Blum et al.,1982; Rubinstein et al., 1986). Therefore, not all the VLDL particles contain apoE. Indeed, apoE associated with VLDL accounts for only 20% of the total VLDL particles in the plasma (Hamilton et al., 1991). It is consistent with the findings that displacement of apoE by apoCII is required for the LPL-catalysed metabolism of plasma VLDL (Kinnunen et al., 1977). Increased plasma level of other exchangeable apolipoproteins such as apoCI or apoCIII may also displace apoE from VLDL particles, as they were found to inhibit human apoE binding to rabbit ß-VLDL (Weisgraber et al., 1990). ß-VLDL referred to a class of ß-migrating lipoproteins (according to the Fredrickson classification), which is also called apoB-containing lipoprotein (Fredrickson & Lees, 1965). Each ß-VLDL particle contains a single copy of hepatic apoB100 or intestinal apoB48 and variable copies of apoE molecules. The presence of multiple copies of apoE enhances the binding affinity of ß-VLDL particle to the receptors through the multiple interactions between apoE and LDLr.

Importantly, HCV has been shown to exploit the VLDL biogenesis pathway as a platform for its morphogenesis (Huang *et al*., 2007). Indeed, small interfering RNA-mediated downregulation of apoB level or treating cells with a MTP inhibitor, resulted in the inhibition

of HCV production, without effects on the infection efficiency and viral RNA replication (Huang *et al*., 2007; Gastaminza *et al*., 2008). Moreover, apoB was considered to be a rate-limiting factor for HCV assembly (Gastaminza *et al*., 2008). However, data from other studies indicated that the apoB level and MTP activity have little or no influence on viral assembly and secretion, whereas apoE was demonstrated to be crucial for HCV production. Specific knock-down of apoE expression reduced both intracellular and extracellular infectious titers, but apoB specific knock-down had no appreciable effect on viral production (Chang *et al*., 2007; Jiang & Luo, 2009; Benga *et al*., 2010). Furthermore, MTP inhibitors blocked apoB secretion, without affecting the release of apoE and virus. However, a decreased virus production was observed when using higher concentrations of MTP inhibitors, mirroring the effect of a reduced apoE secretion (Jiang & Luo, 2009). Moreover, Benga *et al*. found that apoE silencing resulted in an early inhibitory effect on the release of infectious particles and a delayed negative effect on the assembly of infectious particles, indicating the important role of apoE in the assembly and secretion of infectious HCV virions (Benga *et al*., 2010). Finally, reconstitution of HCV assembly in a non-hepatic cell line (293T cells) showed that the only apolipoprotein essential for HCV morphogenesis is apoE (Da Costa *et al*., 2012; Hueging *et al*., 2014).

6. Other apolipoproteins

6.1 ApoCs in lipoprotein metabolism and HCV infection

Human apoCs (including apoCI, apoCII and apoCIII) as constituents of chylomicrons, VLDL and HDL (Table 4), and their different roles in the lipoprotein metabolism have been widely investigated in cell culture and transgenic or gene targeting mice (Jong *et al*., 1999). It was found that apoE-mediated uptakes of TG-rich emulsions by rat hepatocytes and HepG2 cells were strongly inhibited by apoCI and apoCIII (Shelburne *et al*., 1980; Quarfordt *et al*., 1982; Oswald & Quarfordt,1987). Further studies showed that apoCI and apoCII inhibited the apoE-mediated binding of ß-VLDL to LRP by displacing apoE from ß-VLDL particle (Kowal *et al*., 1990; Weisgraber *et al*., 1990; Swaney & Weisgraber, 1994). Interestingly, apoCIII did not affect the binding of ß-VLDL to LRP, but it was reported to completely obstruct the apoB-mediated binding of lipoprotein particles to LDLr through masking apoB receptor domain (Agnani *et al*.,1991; Clavey *et al*., 1995). This inhibitory effect was also observed for apoCII

but not apoCI (Clavey *et al*., 1995). Study in human apoCI transgenic mice has demonstrated that the elevated plasma lipid level was primarily caused by an impaired uptake of VLDL rather than the increased production by the liver or a diminished lipolysis of plasma VLDL (Jong *et al*., 1996; Shachter et al.,1996). While in human apoCIII overexpressing mice, an accumulation of large, TG-rich VLDLs with a mild elevation of plasma cholesterol was observed. This is consistent with an altered LPL-mediated TG lipolysis rather than an impaired hepatic clearance. Interestingly, the impaired clearance of VLDL TGs from the plasma of human apoCIII transgenic mice, can be overcome by cross-breeding the apoCIII transgenic mice with mice expressing human apoE, indicating the interference in apoE-mediated clearance of TG-rich lipoproteins by apoCIII (Aalto-Setala *et al*., 1992; de Silva *et al*., 1994). However, a study in apoCIII-/- mice demonstrated that the enhanced LPL-mediated lipolysis is independent of apoE, suggesting that the hypertriglyceridemia in mice overexpressing human apoCIII is primarily caused by an inhibition on the LPL-mediated lipolysis of VLDL TGs rather than apoE-mediated VLDL clearance (Jong *et al*., 2001).

Table 4. Association of major apolipoproteins and their functions.

Type	CM	VLDL	IDL/CM	LDL	HDL	Function
AI	Ex	Ex			St	LCAT activator
AII	Ex	Ex			Ex	Inhibits HPL
B100		St	St	St		Hepatic VLDL synthesis and secretion, binds LDLr
B48	St		St			CM synthesis,assembly and secreation
CI	Ex	Ex			Ex	Inhibits VLDL binding to LRP, regulates LPL
CII	Ex	Ex				Activates LPL
CIII	Ex	Ex	Ex		Ex	Inhibits LPL
E	Ex	Ex	Ex		Ex	Binds LDLr

The influence of apoCs on the LPL-mediated lipolysis of plasma TG-rich lipoproteins is also important. As already mentioned (Fig.21), apoCII is an essential co-factor in the LPL-mediated hydrolysis. However, at higher concentration it was found to inhibit rather than stimulate LPL activity (Shachter *et al*., 1994). In brief, apoCs play a role in regulating LPL-mediated lipolysis of plasma VLDL remnants, and they participate in the hepatic clearance of lipoprotein remnants through modulating the interactions of apoE and apoB with their receptors. LPL is a member of lipase family which includes pancreatic lipase, hepatic

triglyceride lipase (HTGL) and endothelial lipase (Wong & Schotz, 2002). It is the rate-limiting enzyme for the hydrolysis of triglycerol core of the circulating VLDL, chylomicrons and triglyceride rich-lipoproteins. The products of LPL-catalyzed hydrolysis including non-esterified fatty acids and 2-monoacylglycerol are taken up and utilized by tissues (Mead *et al*., 2002). LPL is therefore a key enzyme involved in the normal lipoprotein metabolism, and LPL deficiency was shown to cause an elevated plasma triglyceride level (termed hypertriglyceridemia) (Okubo *et al*., 2007).

Importantly, LPL and HTGL have been demonstrated to independently reduce HCV infectivity through their catalytic activities on HCV-associated lipoproteins (Shimizu *et al*., 2010). LPL treatment resulted in a shift of HCV particles to higher density fractions, and the association of HCV with apoE was significantly reduced, as the ratio of HCV RNA associated with apoE to total HCV RNA was markedly lower in LPL-treated samples compared to PBS-treated samples. Another study pointed out that LPL inhibits HCV infection by acting on HCV-associated triglyceride-rich lipoproteins mostly through the bridging function of LPL rather than its catalytic activity, since the lipase inhibitor only slightly restored HCV infection (Maillard *et al*., 2011). Indeed, the ‘‘bridging’’ mechanism has already been proposed in an earlier study, in which exogenously added LPL increased the attachment of authentic HCV particles from patient sera to hepatoma cell lines by forming a linker between HCV-associated lipoproteins and cell surface HSPGs. However, this enhanced virus binding was followed by an immobilization of HCV particles at the cell surface, actually leading to an inhibiton of HCV internalization. An anti-LPL antibody, which blocks both the bridging and catalytic functions of LPL, completely abolished the inhibitory effect of LPL on HCV infectivity (Andreo *et al*., 2007). Surprisingly, it was found that the LPL-mediated inhibition of HCV infection is reversed by apoCIII/apoIV in the VLDL, VLDL-LVP and LDL-LVP particles from HCV-postive patients, through modulation of LPL lipolytic activity. Data also showed that plasma HCV RNA level is negatively correlated to plasma LPL lipolytic activity, but has a strong positive relationship with apoCIII quantity in VLDL (Sun *et al*., 2013). Finally, plasma apoCIII has also been proposed as a potential biomarker associated with the outcome of acute HCV infection (Molina *et al*., 2008).

Unlike apoCIII, HCV-associated apoCI was found to function in the binding of viral particles to cell surface HSPGs, and its C-terminal peptides inhibited HCVcc infection, suggesting the important role of C-terminal half of apoCI in HCV infection (Meunier *et al*., 2005; Meunier *et al*., 2008). ApoCI was also found to increase HCVpp infectivity, most likely by promoting the membrane fusion (Dreux *et al*., 2007). Finally, ApoCII has also been shown to be present in LVPs (André *et al*., 2002; Diaz *et al*., 2006; Scholtes *et al*., 2012). Interestingly, in genotype 1 infected patients, apoB and apoCII levels were found to be significantly higher in patients who obtained SVR than in those who did not response to the pegIFN plus RBV therapy (Aizawa *et al*., 2012). However, the exact role of apoCII in HCV infection has not been investigated yet.

6.2 ApoAI in lipoprotein metabolism and in HCV infection

ApoAI is the major protein moiety (70%) in high density lipoprotein (HDL), which circulates in the bloodstream, functioning in the extraction and transport of cholesterol from peripheral tissues to the liver, a process called ‘‘the reverse cholesterol transport (RCT) pathway’’ (Fielding & Fielding, 1995). The high-density lipoprotein cholesterol (HDL-c) is termed as ‘‘good cholesterol’’, as increased plasma HDL level is correlated with a decreased risk of atherosclerosis and cardiovascular disease (Boden & Pearson, 2000; Toth *et al*., 2005). Indeed, the cardioprotective effect of HDL is largely contributed by its surface protein apoAI (Luc *et al*., 2002), which is the principal and most potent activator for Lecithin: cholesterol acyltransferase (LCAT) (Sorci-Thomas *et al*., 2009). LCAT is the key enzyme that catalyzes the esterification of free cholesterol to yield cholesteryl esters in HDL particles, playing a critical role in the metabolism of HDL (Jonas *et al*.,1999; Jonas *et al*., 2000).

ApoAI is mainly synthesized in the liver and the intestine (Kan *et al*., 2000). It is an exchangeable component for CM and VLDL particles, but it is also a structural component of plasma HDL. ApoAI was also found in LDL particles (apoAI-LDL) in the serum from patients with coronary artery diseases (CAD). And apoAI-LDL was thought to be generated by an oxidizing reaction which combines apoAI and LDL. Though the pathophysiological role of apoAI-LDL remains unclear yet, its close association with CAD makes it a potential prognostic marker of CAD (Ogasawara *et al*., 2008). Notably, the association of apoAI and LDL particles is altered in HCV chronic-infected patients, with significantly decreased level

of apoAI in the LDL fraction and no apparent differences in the VLDL and HDL fractions comparing with that of healthy donors. Furthermore, the impaired apoAI/LDL association was demonstrated to occur in the HCV-infected Huh 7.5.1 cells, and cells that replicate HCV subgenomic replicons, indicating that viral replication affects the association of intracellular apoAI and apoB-containing lipoproteins. Conversely, inhibiting the expression of apoAI by RNA interference resulted in a markedly reduced viral RNA replication and HCV particle production in cell culture, demonstrating that apoAI is required for HCV production (Mancone *et al*., 2010). Importantly, apoAI has also been shown to be present at the surface of purified HCV particles (Catanese *et al*., 2013b).

Indeed, earlier studies in patients with HCV chronic infection have already suggested the complicate relationship between HCV and the host lipid and lipoprotein metabolism (Serfaty *et al*.,1991; Cicognani *et al*., 1997; Fabris *et al*., 1997). In infected patients with liver cirrhosis and decreased levels of serum, LDL and total cholesterol were associated with a severe progression of the disease and a negative prognosis (Serfaty *et al*., 2001). Consistent with lower serum apoB or cholesterol level found in HCV chronic-infected patients (Serfaty *et al*., 2001), HCV viral load was found to be inversely correlated with plasma apoB level (Petit *et al*., 2003). The decreased apoB level could be due to an impaired hepatic synthesis of apoB-containing lipoproteins induced by HCV infection or a dysfunctional metabolism of these lipoproteins. A study performed in HCV subgenomic replicon-containing cells demonstrated that the expression of HCV non-structural proteins resulted in a decreased synthesis and secretion of apoB. Moreover, it also reduced the microsomal triglyceride transfer protein (MTP) transcript levels, the promoter activity and MTP protein activity, thus severely affecting the lipidation of apoB (Domitrovich *et al*., 2005). MTP transfers lipid to apoB to assemble a phospholipid-rich precursor, which is the first step of VLDL production. MTP-dependent lipid transfer is also essential for the formation of triglyceride-rich lipid droplets in the ER lumen where apoB-containing precursor particles fuse with these TG-rich droplets to form mature VLDL particles (Gordon & Jamil, 2000; Read *et al*., 2000; Shelness & Sellers, 2001).

In summary, HCV infection is closely linked to the lipid and lipoprotein metabolism in infected hepatocytes. Cellular lipid and lipoproteins are not only required for several steps of the HCV life cycle, but they are also important constituents of infectious HCV particles.

Furthermore, HCV infection directly and indirectly dysregulates the circulating lipoprotein metabolism as well. In the end, HCV infection causes lipid and lipoprotein metabolism disorders such as hepatic steatosis, hypobetalipoproteinemia and hypocholesterolemia, which are the common clinical characteristics found in chronically-infected patients.

III. Cellular factors in HCV entry

1. HCV attachment factors

As discussed above, HCV cell entry is a complicated multiple-step process, involving a variety of cellular factors. Entry begins with the binding of virus to host cell, which is mediated by the interactions between viral particles and attachment factors. The initial attachment step is thought to bind and concentrate viral particles on cell surface, facilitating their interaction with specific receptors/co-receptors, and eventually triggering the internalization of viral particle into the hepatocytes. Different from the true entry receptors for virus infection, attachment factors are usually abundant molecules on various cell surface, and do not play important roles in determining virus-host cell tropism (Jolly & Sattentau, 2013). The presence of virus attachment factors are not necessarily required for infection, but it was found to enable viral infection in permissive cells more efficiently (Lee *et al*., 2001). So far, heparan sulfates proteoglycans and LDLr have been indicated as the initial attachment factors for HCV cell entry into hepatocytes. However, HCV receptor SRB1 was also found to function in the cell attachment, in addition to its role in the specific virus-receptor interactions (Dao Thi *et al*., 2012). The C-type lectin DC-SIGN (Dentritic cell specific ICAM-3 grabbing non-integrin), which has been demonstrated to be the high-affinitiy attachment factor playing an important role in HIV trans-infection (Geijtenbeek *et al*., 2000a; Geijtenbeek *et al*., 2000b), as well as L-SIGN (liver/lymph node-derived specific ICAM-3 grabbing non-integrin) were also found to be capable to capture HCV particles, suggesting their potential contribution in the establishment of HCV persistent infection and HCV-related immunopathogenesis (Gardner *et al*., 2003; Pohlmann *et al*., 2003; Lozach *et al*., 2004). Here, we focus on the role of HSPG attachment factor in HCV infection.

1.1 HSPGs

HSPGs were considered to be involved in HCV absorption, because treatment of HCV particles with heparin, a homologue of heparan sulfate, caused a significant inhibition of viral attachment to hepatoma cells (Germi *et al*., 2002). Treating hepatoma cells with heparinases, which remove cell surface heparan sulfates, also resulted in an apparently decreased HCV infection (Koutsoudakis *et al*., 2006), as well as a significant reduction of HCV particles binding to Huh-7 cells (Morikawa *et al*., 2007). To better understand how HSPGs function in HCV cell-surface attachment, basic information about HSPGs is stated in the last part of the introduction section.

1.2 LDLr

LDLr is an 839 aa transmembrane glycoprotein, responsible for the uptake of cholesterol-containing lipoprotein particles into cells, playing a crucial role in cholesterol homeostasis (Brown & Goldstein, 1986). The primary ligand of LDLr, as the name suggests, is LDL. Serum LDL particles contain triacylglycerols, cholesteryl esters and phospholipids and carry one-copy apoB100 molecule as their major protein component present on the surface, mediating the interaction with LDLr (Clavey *et al*., 1991). Besides apoB, LDLr also binds with high affinity to lipoproteins that contain multiple copies of apoE molecules such as ß-VLDL (Yamamoto *et al*., 2008). Furthermore, the endocytic activity of LDLr was demonstrated in certain cell types such as hepatocytes and lymphocytes (Garcia *et al*., 2001; He *et al*., 2002).

Interaction between HCV particles and lipoproteins led to the suggestion that the LDLr is a potential attachment factor for HCV (Burlone & Budkowska, 2009). Early studies demonstrated that LDLr can mediate HCV virion absorption on Cos-7 or Vero cells, since anti-LDLr or LDL/VLDL were shown to significantly inhibit HCV-cell binding (Monazahian *et al*., 1999; Germi *et al*., 2002). Furthermore, LDLr-mediated endocytosis of HCV was obtained by the finding that anti-LDLr antibodies at a high concentration can completely inhibit HCV endocytosis into both hepatoma cell line HepG2 and B lymphocyte line G4 (Agnello *et al*., 1999). A more recent study confirmed the potential role of LDLr in HCV entry into primary human hepatocytes (Molina *et al*., 2007). It was then suggested that LDLr serves as a co-receptor supporting viral entry by interacting with apoE ligand which is associated with an infectious HCV/lipoprotein complex (Owen *et al*., 2009; Felmlee *et al*.,

2013). Very recently, the involvement of LDLr in HCV infection was further explored. It was demonstrated that LDLr is not essential for infectious HCV particle entry, but plays an important role in the optimal viral RNA replication through its physiological function (Albecka *et al*., 2012). Furthermore, interaction between HCV particles and LDLr would rather lead to a non-productive entry pathway. Then, it was also demonstrated that HCV infection stimulates LDLr expression in both HCV-infected Huh7 cells and in liver tissue from chronic HCV-infected patients. Increased expression of LDLr was shown to promote lipid uptake of HCV-infected cells, therefore favoring viral proliferation (Syed *et al*., 2013).

2. HCV entry receptors /co-receptors

2.1 CD81

The first proposed HCV receptor is CD81, as it was shown to be sufficient for binding not only the recombinant viral glycoprotein E2 but also HCV particles from the serum of challenged chimpanzees (Pileri *et al*., 1998). The crucial role of CD81 in HCV entry was soon afterwards confirmed by the findings that HCVpp infection in human hepatocytes (PLC/PR5) or hepatoma cell lines Huh-7 was blocked by anti-CD81 antibodies or a recombinant CD81 molecule (Bartosch *et al*., 2003a; Hsu *et al*., 2003). Furthermore, HCVpp entry was inhibited in the CD81-silenced Huh7 cells, and CD81-negative HepG2 cells turned to support HCVpp infection only after exogenous expression of CD81 (Bartosch *et al*., 2003b; Zhang *et al*., 2004). Further studies demonstrated that only HCV entry but not binding is CD81-dependent, indicating that CD81 actually is a HCV co-receptor acting after virus attachment (Cormier *et al*., 2004; Flint *et al*., 2006).

CD81 is a 26 kDa integral membrane protein, a member of the tetraspanin family, which are characterized by their four transmembrane domains, conserved CCG motif and 2-6 cysteine residues, also by exerting their functions through clustering multiple partner proteins to form the tetraspanin-enriched microdomains (TEMs) (Hemler *et al*., 2003). TEMs have been demonstrated to play important roles in many cellular processes including cell adhesion, migration, proliferation, differentiation and cell signalling (Hemler *et al*., 2001; Levy & Shoham, 2005; Berditchevski & Odintsova, 2007), as well as in pathogen infections (Monk & Partridge, 2012). Like all tetraspanins, CD81 contains four-transmembrane domains, short

cytoplasmic domains and two extracellular loops EL1 and EL2, which are also named as the small extracellular loop (SEL) and the large extracellular loop (LEL) (Maecker *et al*., 1997; Levy *et al*., 1998) (Fig.26).

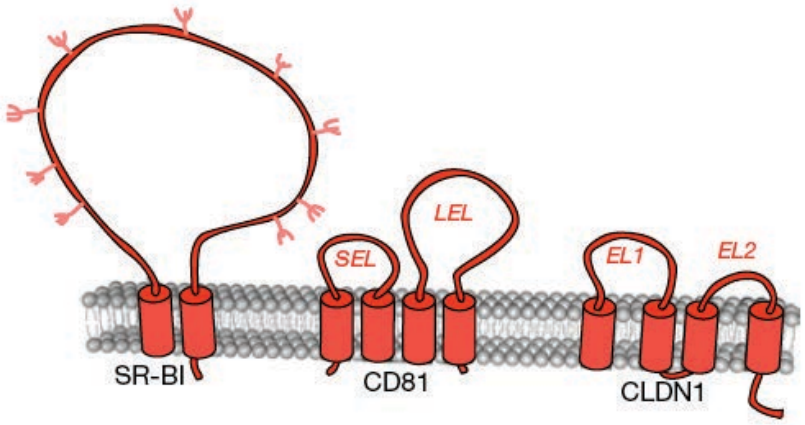


Fig. 26: Shematic illustration of SRB1, CD81 and CLDN1

The transmembrane domains of each molecules are presented as red rods. The glycosylation sites in the extracellular domain of SRB1 are shown in the shape of light red trees. The nomenclature of the extracellular domains of CD81 and CLDN1 are indicated in the abbreviations SEL (Small Extracellular loop), LEL (Large Extracelluar Loop), and EL1 (Extracellular Loop1), EL2 (Extracellular Loop 2).

A truncated soluble form of HCV glycoprotein E2 ($E2_{661}$, aa384-661, lacking the transmembrane domain) was shown to bind specifically to cell surface CD81 but not other members of the tetraspanin family (Flint *et al*., 1999a). It was also found that binding to CD81 required the conformational integrity of E2, which involves multiple epitopes in the glycoprotein E2 (Flint *et al*., 1999a; Patel *et al*., 2000; Hadlock *et al*., 2001). A study performed with pseudotyped virion incorporated with E1E2 showed that a conserved G436WLAGLFY motif of E2 was the determinant for CD81 binding and function in viral entry (Drummer *et al*., 2006). Functional analysis of E2 proteins from HCV 1a and 1b isolates revealed that at least two different domains within E2 are involved in the interaction with CD81. The first domain located around aa 613-618 of E2 was essential for CD81 binding, while the second domain involving two hypervariable regions (HVR1, aa 384-410 and HVR2, aa 461-480) were demonstrated to modulate E2 binding to CD81 (Roccasecca *et al*., 2003). Importantly, deletion of HVR1 did not affect E1E2 heterodimerization and the conformation of E2, whereas the HVR1-deleted recombinant E2 displayed an increased CD81 binding,

which indicated the potential masking of E2 region for CD81 recognition by HVR1 (Forns *et al*., 2000; Roccasecca *et al*., 2003; McCaffrey *et al*., 2011). This observation was confirmed by the finding that HVR1-deleted virus are more sensitive to anti-CD81 antibody neutralization and soluble hCD81-LEL competition (Bankwitz *et al*., 2010). Though the entry of HVR1-deleted HCVcc was severely disrupted, a single adaptive mutation at the position 415 (N415D) can fully restore the infectivity of HVR1-deleted virus (McCaffrey *et al*., 2011). These data suggested that HVR1 is not crucial for CD81 binding in the context of HCVcc infection, and the HVR1-deleted virus carrying N415D mutation probably binds to CD81 with high affinity (McCaffrey *et al*., 2011). Consistently, introduction of N415D into the wild-type JFH1 genome resulted in an increased sensitivity of the mutant virus ($JFH1_{\mathbf{N415D}}$) to the neutralization by hCD81-LEL molecules and anti-CD81 antibodies (Dhillon & Rychłowska, 2010).

In the past decades, extensive studies using linear or conformational antibodies against E2 of different HCV isolates have been done to evaluate the neutralizing antibodies (nAbs) that can efficiently prevent E2-CD81 interaction, thus to determine the crucial CD81-binding domains of E2 (Allander *et al*., 2000; Hadlock *et al*., 2000; Schofield *et al*., 2005; Owsianka *et al*., 2006; Keck *et al*., 2008; Keck *et al*., 2011; Zhao *et al*., 2014). For instance, the broadly neutralizing antibody AP33 which recognizes the conserved, linear epitope spanning the aa 412-423 of E2, was shown to potently inhibit the interaction of E2 with CD81 (Owsianka *et al*., 2001; Owsianka *et al*., 2005; Owsianka *et al*., 2006). This region (aa 412-423) of E2 comprises the highly conserved residue W420, together with other specific amino acids including Y527, W529, G530 and D535 that were shown to be conserved across all HCV genotypes, playing a critical role in the CD81 binding of E2 (Owsianka *et al*., 2006). With the successful crystallization of E2, CD81 binding sites in E2 of HCV strain H77 were demonstrated to be located in the front layer (aa 421-453) and the CD81 receptor binding loop (aa 519-535) (Kong *et al*., 2013; Khan *et al*., 2014). Furthermore, the authors of this study also showed that a single mutation at position 427 (L427Y) in the front layer or addition of glycosylation at position 442 or 428 led to a totally abrogated CD81 binding (Kong *et al*., 2013).

The binding site for E2 was mapped to the large extracellular loop of CD81 (residues 113-201), in which the conserved cysteine residues and several other amino acids were shown to be important for high-affinity E2 binding (Pileri *et al*., 1998; Flint *et al*., 1999b; Patel *et al*., 2000; Drummer *et al*., 2002; Flint *et al*., 2006). Interestingly, CD81 LEL from tamarin (tCD81-LEL) was also able to bind E2 and displayed stronger affinity than human CD81 molecule (hCD81-LEL), indicating that binding of E2 to CD81 has no correlation with the species permissiveness to HCV infection (Meola *et al*., 2000). Ectopic expression of chimeric molecules CD9/CD81 (CD9 is also a member of the tetraspanin family) in the HepG2 cell line, which is negative for CD81 expression, endowed the permissivity of HCVpp infection in the transfected cells. This data confirmed the requirement of CD81 for viral glycoprotein-mediated infection and the determinant role of CD81 LEL in viral entry (Zhang *et al*., 2004). In contrast to the direct role of CD81 LEL in E2 binding, the SEL of CD81 was reported to play an indirect role in the interaction with HCV by contributing to the optimal cell surface expression of CD81 LEL (Maciopinto *et al*., 2001). Other regions of CD81 including the intracellular C-terminal and the transmembrane domain were not required for the soluble E2 binding but were required for HCVpp entry (Bertaux & Dragic, 2006). It has been well demonstrated that the palmitoylation of intracellular cysteine residues is essential for the oligomerization of CD81, as well as the interaction with other proteins and the partition of cholesterol-riched microdomains on cell surface (Charrin *et al*., 2002; Charrin *et al*., 2003; Soldaini *et al*., 2003; Cherukuri *et al*., 2004a; Cherukuri *et al*., 2004b; Delandre *et al*., 2009). A recent study showed that the palmitoylation modification of CD81 strongly promoted HCV entry, partly by modulating the association of CD81 with other tetraspanins at cell surface (Zhu *et al*., 2012). However, the association of CD81 with tretraspanin-enriched microdomains has been previously reported not to be essential for HCV entry (Rocha-Perugini *et al*., 2009). With a growing number of cellular factors involved in HCV entry process being identified, the mechanism of CD81 in viral entry gets partly elucidated. By using pharmacological inhibitors and small interfering RNAs, it was found that CD81 engagement induced Rho family GTPase-dependent actin rearrangements, allowing lateral movement of viral particle to the area of cell-to-cell contact where E2-CD81 complexes encounter tight junction (TJ) proteins OCLN, ZO-1 and CLDN1 (Brazzoli *et al*., 2008). Both CLDN1 and OCLN were described as HCV co-receptors required for the late step in viral entry (Evans *et al*., 2007; Ploss *et al*., 2009). Furthermore, the association of CLDN1 with CD81 was shown to be essential for HCV entry (Harris *et al*., 2008; Harris *et al*. 2010;

Bonander *et al*., 2011). OCLN was demonstrated to act late in the HCV entry process, during a post-binding step and after the co-receptor association between CD81 and CLDN1 (Ploss *et al*., 2009; Sourisseau *et al*., 2013). Altogether, these findings revealed that HCV receptor CD81, more than just being a cell surface target for viral binding, plays a fundamental role in HCV infection by actively inducing endogenous cellular events that coordinate the full virus-receptor interactions during the binding and post-binding steps in HCV entry process.

Importantly, mouse CD81 molecule failed to bind soluble E2, but it supported both HCVpp and HCVcc infection, indicating that the interaction between soluble E2 and CD81 LEL or full-length molecule can only partially reflect the entry of authentic HCV particle (Flint *et al*., 2006; Bitzegeio *et al*., 2010). Moreover, the adaptation of HCV particles to mouse CD81 led to a lower dependence of HCV on human SRB1 and OCLN, other two HCV entry factors (Bitzegeio *et al*., 2010). Apart from the role of CD81 in viral entry, it has been suggested that this tetraspanin is also required for efficient viral RNA replication (Zhang *et al*., 2010).

Another important aspect of CD81 in HCV infection is its controversial role in the viral cell-to-cell transmission route. Both CD81-dependent and CD81-independent cell-to-cell spread have been reported in the past years (Timpe *et al*., 2008; Witteveldt *et al*., 2009; Brimacombe *et al*., 2011). Moreover, CD81-independent uptake of HCV particles was demonstrated in myeloid dendritic cells (mDCs) which express extremely low levels of CD81 and SRB1. This finding indicated that CD81-independent cell entry of HCV may exist (Lambotin *et al*, 2010). CD81 was initially reported not to be necessary for cell-cell transmission, because HCV infection in Huh7.5 cells was only partially inhibited by soluble CD81 molecules and anti-CD81 antibodies. Additionally, cell lines that expressed little or no CD81 such as Huh7.5/CD81-knocked-down cells, Huh7 Lunet cells or HepG2 cells, were found to support HCV cell-cell transmission, though they were non-permissive to cell-free infection (Timpe *et al*., 2008; Witteveldt *et al*., 2009). Also, mutant viruses JFH1$_{\mathbf{W529A}}$ and JFH1$_{\mathbf{D535A}}$ that have low affinity for CD81 binding were demonstrated to be competent for cell-to-cell spread in cell culture (Witteveldt *et al*., 2009). However, another study demonstrated that HCV minimal receptor complex including CD81, SRB1, CLDN1 and OCLN are essential not just for the entry of cell-free virus, but also the direct cell-cell transmission of HCV. The authors explained that the observed HCV cell-cell transmission in CD81-negative HepG2 cells was probably due to the false-positive results caused by cell aggregates between HCV target and

producer cells (Brimacombe *et al*., 2011). More recently, a study demonstrated that CD81 knockdown, which completely blocked HCV cell-free infection, still supported cell-to-cell transfer between HCV producer and target cells, and CD81 levels on the producer cells were indicated to modulate the efficiency of cell-cell transmission (Catanese *et al*., 2013a). This finding confirmed the possibility that the CD81-independent HCV cell-cell transmission route exists, as well as the CD81-dependent cell-cell spread.

As CD81 is ubiquitously expressed by almost all the cell types except red blood cells, platelets and neutrophils (Rubinstein *et al*., 1996; Hemler *et al*., 2001), it is impossible to be the sole factor determining HCV human liver tropism. However, hepatocytes do not express the CD81 partner EWI-2 wint, which was shown to inhibit the interaction between HCV envelope glycoproteins and CD81 *in vitro*, and also to be able to affect the colocalization of CD81 with CLDN1 (Rocha-Perugini *et al*., 2008; Montpellier *et al*., 2011; Potel *et al*., 2013). Therefore, the absence of a natural inhibitor of HCV-CD81 interaction at the cell surface of hepatocytes could account for the hepatotropism of HCV, providing a novel mechanism for host factors determining the tropism of a pathogen.

Notably, in different cell lines, CD81 promiscuously associates with various cell-surface proteins including other tetraspanins, forming a variety of molecular complexes that function in both physiological and pathological processes (Imai & Yoshi, 1993; Levy *et al*., 1998; Radford *et al*., 1996; Maecker *et al*., 1998). For example, in T cells CD81 associates with T-cell specific molecules such as CD4, CD8 and other molecules including integrin alpha 4 beta 1 (CD49d/CD29) and CD82 (Imai & Yoshi, 1993; Mannion *et al*., 1996). whereas in B cells CD81 is known to cluster with CD19, CD21 and Leu-13 (Bradbury *et al*., 1992; Fearon & Carter, 1995). Binding of HCV envelope glycoprotein E2 to the CD81 molecule on these cells of the immune system could have serious consequences. It has been reported that E2 binding to CD81 provided a co-stimulatory signal for T cells, resulting in significant production of interferon gamma (IFN-γ) and tumor necrosis α (TNF-α) by liver TCRγδ+ T cells, which could be an important factor in the liver damage caused by HCV infection (Wack *et al*., 2001; Tseng *et al*., 2001). Also, binding of E2 to CD81 has been demonstrated to inhibit the production of IFN-γ by natural killer (NK) cell, offering a possible mechanism for HCV altering host innate immune defenses to favor the establishment of persistent infection (Tseng & Klimpel, 2002; Crotta *et al*., 2002). However, a later study showed that HCV envelope

proteins did not affect NK cell functions in the context of infectious viral particles (Yoon *et al*., 2009). The engagement of CD81 on B cells by HCV envelope proteins has been shown to preferentially activate naïve B lymphocyte proliferation, which may explain the high rate of B lymphocyte disorders such as cryoglobulinemia and B cell lymphomas in chronic HCV patients (Agnello *et al*., 1992; Clifford *et al*., 1995; Chan *et al*., 2001; Quinn *et al*., 2001; Rosa *et al*., 2005). It was also shown that binding of HCV E2 to CD81 on human hepatic stellate cell (HSC) surface increases the expression of matrix metalloproteinase-2 (MMP-2) (Mazzocca *et al*., 2005). Consequently, enhanced degradation of the hepatic extracellular matrix could lead to accelerated inflammatory infiltrations and further parenchymal damage in HCV-infected liver.

2.2 SRB1

SRB1 was discovered as an HCV receptor by the finding that HCV soluble E2 protein can bind to human heptoma cells independently of the previously proposed HCV receptor CD81. The protein responsible for E2 binding on the HepG2 cells was identified to be the SRB1 molecule (Scarselli *et al*., 2002). SRB1 is a 82 kDa cell surface glycoprotein, consisting of cytoplasmic N-terminal and C-terminal tails and two transmembrane domains that are spearated by a large heavily N-glycosylated extracellular loop (ECL) (Fig.26). SRB1 is primarily expressed in tissues with important roles in cholesterol metabolism, such as the liver, ovaries and adrenal glands (Pittman & Steinberg, 1984; Glass *et al*., 1985; Rinninger & Pittman, 1987; Jackle *et al*., 1993; Rinninger *et al*., 1994). Physiologically, SRB1 was first identified as the HDL receptor, mediating selective uptake of HDL cholesterol (Trigatti *et al*., 1999; Liadaki *et al*., 2000; Rigotti *et al*., 2003; Rhainds & Brissette, 2004). In addition to HDL, SRB1 can also interact with a variety of ligands including native LDL, acylated or oxidized LDL (AcLDL, oxLDL), VLDL and phospholipids (Krieger *et al*., 1999; Krieger *et al*., 2001). The selective uptake of HDL cholesterol by SRB1 is very important for the clearance of cholesterol from the plasma. In the bloodstream, HDL functions as a vehicle for uptake and transport of the excess of cholesterol from the peripheral tissues to liver for further metabolism (Tall *et al*., 1998; Trigatti *et al*., 2000). This process is called ‘‘reverse cholesterol transport’’, and depends on the HDL receptors expressed on the surface of hepatoctyes, which includes the high-affinity HDL docking receptor SRB1 and the endocytic receptor, the beta-chain of ATP synthase (Martinez *et al*., 2003). Alteration of SRB1 activity was shown to have a corresponding effect on the cholesterol metabolism *in vitro* and *in vivo* (Trigatti *et al*., 2000;

Rigotti *et al*., 1997; Temel *et al*., 1997). Notably, SRB1 partner PDZK1, a PDZ domain-containing cytosolic protein was found to bind the C-terminus of SRB1 and control the expression of SRB1 in a tissue specific, post-transcriptional fashion (Ikemoto *et al*., 2000; Kocher *et al*., 2003). Another study showed that PDZK1 was required for maintaining SRB1 steady state level in the liver but not its surface localization or function (Yesilaltay *et al*., 2006). Interestingly, SRB2, an altenative form of SRB1 produced from a spliced variant of SRB1 mRNA also exists, which differs from SRB1 in the C-terminal domain (Webb *et al*., 1997; Webb *et al*., 1998). It has been shown that, as compared to SRB1, SRB2 internalized HDL protein more rapidly, however its selective cholesteryl-ester uptake was significantly lower, suggesting that SRB2 may affect cellular cholesterol homeostasis in a manner different from that of SRB1 (Eckhardt *et al*., 2004).

The interaction of HCV sE2 with human SRB1 was found to be very specific, as neither mouse SRB1 nor another closely related human scavenger receptor hCD36 bound to sE2 (Tao *et al*., 1996; Scarselli *et al*., 2002). Furthermore, HVR1 of E2 plays a major role in E2-SRB1 interaction (Scarselli *et al*., 2002; Bartosch *et al*., 2003a). Coincidentally, a physiological ligand of SRB1, oxLDL, was shown to potently inhibit HCV entry, but it did not affect the binding of soluble E2 to SRB1 (von Hahn *et al*., 2006). Intriguingly, another natural ligand for SRB1, HDL was shown to specifically enhance HCV entry (Meunier *et al*., 2005; Voisset *et al*., 2005; Voisset *et al*., 2006; Dreux *et al*., 2006; Catanese *et al*., 2007). This HDL-mediated enhancement of HCV infectivity was demonstrated to be largely dependent on the presence of of SRB1 and E2 HVR1 (Voisset *et al*., 2005). Furthermore, drugs that inhibit the lipid exchange activity of SRB1, which mediates HDL cholesteryl transfer, were also able to abrogate the active effect of HDL on HCV entry, suggesting that the HDL-mediated infection-enhancement probably relies on SRB1 lipid tranfer function (Voisset *et al*., 2005).

The putative role of SRB1 in HCV infection seemed far from being well-defined. Study showed that binding of HCV from sera of infected patients to SRB1 that was functionally expressed on CHO cell surface, was not sensitive to anti-HCV such as anti-E2 and anti-HVR1 antibodies, but was effectively blocked by anti-ß lipoprotein antibodies or by apoB-containing lipoproteins, especially by VLDL. This finding suggested that binding of HCV particle to SRB1 may be mediated by the interaction between SRB1 and HCV-associated lipoproteins (Maillard *et al*., 2006). Further investigation through expressing different SRB1 mutants in rat

hepatoma cells and human background SK-Hep1 cells that both did not express detectable SRB1, and by performing a panel of SRB1 functional complementation assays, demonstrated that SRB1 serves as an HCV entry factor by providing the cell surface binding sites, as well as the physiological lipid transfer functions hijacked by HCV to favor its infection. Also, the C-terminal cytoplasmic tail of SRB1 was shown to modulate the basal HCV entry process without affecting HDL-mediated infection-enhancement (Dreux *et al*., 2009). SRB1 partner PDZK1 was also shown to be necessary for efficient HCV infection in hepatocytes (Eyre *et al*., 2010).

SRB1 was originally proposed to act at an early phase of HCV entry, since kinetics of inhibition of HCVcc infection showed that anti-SRB1 monoclonal antibody MAb C167 could no longer inhibit HCV infection if it was added after virus binding. While, by contrast, the anti-CD81 MAb had the similar inhibitory effect irrespective of when it was added: before or during or immediately after virus binding to cell surface. This data suggested that SRB1 probably was involved in a post-binding step, immediately after viral attachment but before interaction with CD81 (Catanese *et al*., 2010). However, SRB1 and CD81 do not seem to function separately in the process of HCV entry. It has been shown that HCV E2 linked the soluble CD81 and SRB1 protein (Heo *et al*., 2004; Heo *et al*., 2006), suggesting the formation of a heterotrimeric E2-CD81-SRB1 complex which could trigger a series of conformational changes in HCV envelope proteins, and thereby facilitate viral entry and fusion with a cellular membrane.

A study by using anti-SRB1 or anti-CD81 antibody alone, or in combination to neutralize HCV infection showed that HCVcc infection was significantly reduced when cells were incubated with a combination of these antibodies. This synergistic neutralizing effect of the combination of anti-SRB1 and anti-CD81 antibodies suggested that SRB1 and CD81 function cooperatively during HCV infection (Kapadia *et al*., 2007). MßCD depletion of cholesterol from Huh7 cells resulted in an inhibited HCV infection without influence on viral RNA replication and particle secretion. Furthermore, MßCD depletion of cholesterol was also found to alter the membrane expression of CD81 and SRB1, with a decreased cell surface CD81 expression but an increased SRB1 expression. Together, these data suggested that membrane cholesterol has an important effect on HCV entry, possibly through regulating cell surface expression and localization of CD81 (Kapadia *et al*., 2007). Importantly, SRB1 was

shown to regulate the organization of CD81 at the plasma membrane by providing necessary cholesterol for the tetraspanin microdomains assembly, which had an important impact on the hepatocyte permissiveness to plasmodium sporozoite infection (Yalaoui *et al*., 2008).

More recently, it has been reported that HCV particle subpopulations utilize SRB1 for their entry into hepatocytes in several ways. SRB1-mediated entry of HCV particles was demonstrated to be independent of their buoyant densities. While, SRB1 mediated primary attachment of virus subpopulations was found to differ in their buoyant densities, involving the lipoprotein components of HCV particles. For instance, the intermediate density HCVcc particles were demonstrated to utilize SRB1 in an E2-independent, HVR1-independent manner but via a lipoprotein component of viral particle, probably via apoE for virus-cell attachment (Dao Thi *et al*., 2012). However, it seemed to be conflicting with the recent finding that specific siRNAs targeting SRB1 did not affect HCVcc attachment (Jiang *et al*., 2012). Intriguingly, the lipid transfer activity of SRB1 was found to be required for SRB1 mediated entry of all HCVcc subpopulations, independent of the E2-SRB1 interaction (Dao Thi *et al*., 2012). Briefly, previous studies have demonstrated the significant involvement of SRB1 in HCV entry at an early phase, the initial attachment and binding. Nevertheless, recent studies suggested that SRB1 also functinons in the postbinding step of HCV entry, closely linked to CD81 (Syder *et al*., 2011; Zahid *et al*., 2013). It was further considered that the interplay with SRB1 probably delipidate HCV-associated lipoproteins and induces conformational changes of HCV E2, therefore exposing the CD81-binding site and facilitate the following HCV-receptor interactions (Lindenbach & Rice, 2013).

Distinct requirements of SRB1 in HCV cell-free versus cell-to-cell transmission has also been recently described. Though SRB1 is essential for cell-free HCV entry, single mutation in HCV E2 at the position 451 (G451R) was shown to have a reduced dependence on SRB1, which paralleled a decreased sensitivity to the neutalization by anti-SRB1 serum (Grove *et al*., 2008). Coincidentally, an adapted HCV clone showed a significantly decreased dependence on SRB1 for HCV cell-to-cell spread, whereas its parental genome required high levels of SRB1 (Catanese *et al*., 2013a). The relatively more prominent role of SRB1 in HCV cell-to-cell transmission route has been suggested, compared to other HCV receptors including CD81, CLDN1 and OCLN. Thus, the significance of SRB1 in both HCV cell-free infection and cell-cell spead, could provide an effective target for controlling the initial HCV

infection and subsequent viral dissemination in the liver (Brimacombe *et al*., 2011; Syder *et al*., 2011; Zahid *et al*., 2013).

2.3 Tight junction proteins

2.3.1 CLDN1 and OCLN

The discovery of CD81 and SRB1 as HCV receptors was a great turning point in the understanding of HCV entry into host cells. However, CD81 and SRB1 were proven to be insufficient for rendering the non-human hepatic cells susceptible to HCV infection, indicating the requirement of additional factors for productive HCV infection (Hsu *et al*., 2003; Zhang *et al*., 2004; Bartosch *et al*., 2003b). By using an iterative expression cloning and screening approach, the Rice's group successively identified two tight-junction proteins, CLDN1 and OCLN, as HCV co-receptors required for a late step in the process of HCV entry (Evans *et al*., 2007; Ploss *et al*., 2009). However, neither CLDN1 nor OCLN were considered as a classic HCV receptor, since both of them did not seem to directly bind soluble HCV E2 or infectious viral particles (Evans *et al*., 2007; Krieger *et al*., 2010). However, more recently, it was proposed that the EL1 of CLDN1 can interact with E1E2 complex but not soluble E2 (Douam *et al*., 2014).

CLDN1 is a 21 kDa integral membrane protein, belonging to the CLDN superfamily within which the protein is composed of four transmembrane domains (four-TM). CLDN proteins comprise the junctional strands, forming the cellular tight junctions (TJs) (Morita *et al*., 1999; Furuse *et al*., 2010). Though CLDN1 has a similar membrane topology as CD81 (Fig.41), it is not classed into the tetraspanin family, as it lacks the sequence homology and key structural features of the tetraspanins, especially the highly conserved CCG motif in the extracellular loop. Similarly, OCLN, another tight junction protein, is also composed of four membrane spanning domains seperated by two extracellular loops (EL1 and EL2), a N- and C-terminal cytoplasmic terminal tail (Fig.27) (Furuse *et al*., 1998; Paris *et al*., 2008). Interestingly, both full-length and C-terminal truncated OCLN can localize to the TJ. However, the C-terminus of OCLN was suggested to be essential for the correct TJ assembly and funtion (Chen *et al*., 1997).

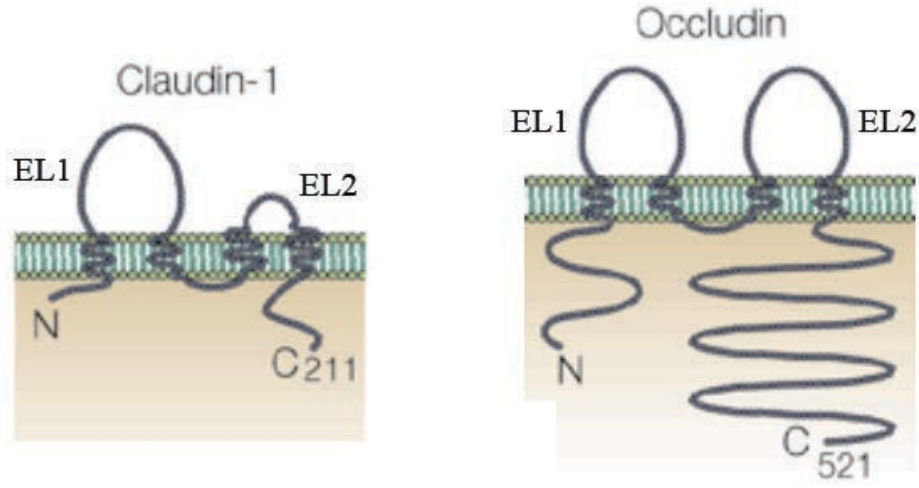


Fig.27: Diagrams of CLDN1 and OCLN membrane topology
Adapted from (Tsukita *et al*., 2001; Yamazaki *et al*., 2011)

The EL1 of CLDN1 is larger than its EL2, while for OCLN the two extracellular loops are of similar size, and its C-terminal tail is relatively longer than its N-terminal region.

CLDNs and OCLNs are widely expressed in many tissues and integral proteins of the tight junctions. The tight junction strands form a paracellular barrier that allows the selective passage of solutes, and maintain cell polarity through separating the apical membrane domains from the basolateral membrane domains (Furuse *et al*., 2010). In the liver, hepatocyte TJs seal the bile canaliculi, organising the intercellular barriers between bile and bloodstream (Easter *et al*., 1983) (Fig.28).

Surprisingly, in hepatocytes or other polarized hepatoma cells, a proportion of CLDN1 molecules is also found to localize outside the TJs (Suzuki *et al*., 2007; Harris *et al*., 2008). Similarly, not all OCLNs reside at the TJs. In epithelial cell lines, highly phosphorylated OCLN molecules were selectively concentrated at the TJs, while those non- or less-phosporylated OCLN molecules appeared in the cytoplasm (Sakakibara *et al*., 1997; Andreeva *et al*., 2001) Importantly, it is the non-junctional pools of CLDN1 that interact with CD81 and are involved in HCV entry process (Harris *et al*., 2010). By using fluorescent-labeled entry factors and fluorescence resonance energy transfer (FRET) methodology, it was shown that anti-CLDN1 antibodies, inhibiting CD81-CLDN1 associations without affecting TJ integrity, have an inhibitory effect on HCV infection. This finding further supported the important role of the complex of CD81 with non-junctional CLDN1 in HCV entry (Krieger *et al*., 2010).

Besides CLDN1, other CLDNs, namely CLDN-6 and -9 were shown to function equally well as CLDN1 in supporting the internalization of HCVpp into human non-hepatic cells ectopically expressing these proteins (Meertens *et al*., 2008). However, specific mAbs against CLDN-6 or CLDN-9 do not inhibit HCV infection in Huh7.5.1 cells or PHH cells despite their inhibitory activity in 293T cells ectopically expressing these CLDNs (Fofana *et al*., 2013). Also, an isolate-dependent usage of CLDNs for HCV cell entry has been reported very recently. It was found that Huh7.5 cells expressing CLDN1 were permissive to all tested HCV isolates, whereas HuH6 cells, another hepatoma cell line, which mainly express CLDN-6 were only susceptible to some HCV strains. Furthermore, viruses with broader CLDN tropism (infecting both CLDN1 or CLDN-6 expressing hepatoma cells) were only partially neutralized by anti-claudin-1 specific antibodies (Haid *et al*., 2014).

2.3.2 Hepatocyte polarity in HCV infection

The involvement of TJ proteins in HCV entry raised the question of the role of hepatocyte polarity for HCV infection (Meertens *et al*., 2008; Harris *et al*., 2010). The importance of membrane polarity in hepatocytes was reported for the infections of other liver tropic pathogens such as plasmodium (Yalaoui *et al*., 2008) and HBV (Bhat *et al*., 2011; Schulze *et al*., 2012). As it is well-known, hepatocytes are the primary epithelial cells in the liver, and they are highly polarized. The plasma membranes of hepatocytes are separated by the TJs into sinusoidal-basolateral and canalicular-apical domains (Fig.43, ‘‘Hepatocyte polarization’’ in green highlight) (Wang & Boyer, 2004). A normal membrane polarity is very critical for hepatocytes to execute their physiological functions, including bile secretion into the bile canaliculi and simultaneous secretion of large quantity of serum proteins into the blood from the sinusoidal-basolateral domains. Many pathological conditions can be induced by the alteration or the loss of hepatocellular polarity (Landmann *et al*., 1995). Recently, it was demonstrated that HCV infection reduced the hepatocyte polarity in a vascular endothelial growth factor (VEGF)-dependent pathway (Mee *et al*., 2010). Treating polarized hepatoma cell line HepG2 cells with VEGF reduced the TJ integrity, suggesting that VEGF negatively regulates TJs integrity and hepatocyte polarity. Furthermore, earlier studies have suggested that HCV infection induced the expression of VEGF in Huh7 cells (Nasimuzzaman *et al*., 2007). This hypothesis was further confirmed by the observation that HCV infection of HepG2, Huh7.5, and PHH cells induced VEGF expression. Other data also showed an inverse

correlation between HepG-CD81 polarization and HCVpp entry. It was found that agents such as phorbol ester that reduced TJ integrity and ablated HepG2-CD81 cell polarization, had a stimulatory effect on HCV entry, while stimulating HepG2-CD81 polarity by PKA significantly reduced HCV entry (Mee *et al*., 2009). Consistently, it was demonstrated that hepatocellular polarization limits CD81 lateral diffusion, thus the movement of HCVpp particles on the surface of infected cells (Harris *et al*., 2014). These findings suggested that hepatocyte polarity restricts HCV infection *in vivo*, thus posing the question of the relevance of the *in vitro* cellular models used to study hepatocyte polarity to closely mimic the *in vivo* authentic HCV infection (Decaens *et al*., 2008).

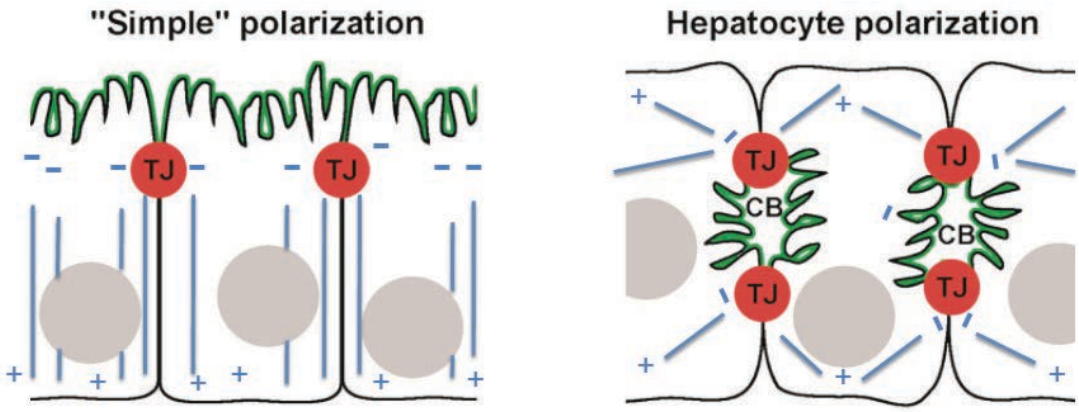


Fig.28: Schemetic illustrations of ‘‘simple’’ epithelial and hepatocyte polarization
Adapted form (Benedicto *et al*., 2012)

Simple epithelia (nonstriated epthelia) is composed by a single layer of divided cells. Their basal domains are attached to the underlying basement membrane, and their apical membranes face the organism’ exterior such as the luminal space in the case of the intestinal lining. In contrast, the hepatocyte polarization is more complicated. Hepatocytes distinguish them form the simple epithelia by their polygonal multipolar organization with at least two basal surfaces facing the endothelial lining, and two apical surface forming the intercellular enclosed BC between adjacent cells. They are rich in TJs, which are important for regulating the exchange between cells, and maintaining the polarity of hepatocytes (Perrault & Pecheur, 2009; Treyer & Musch, 2013). TJs (in red) represent the barrier located at the apical domain (in green highlight) and basolateral domain of the cell membrane. BC: bile canaliculus.

2.4 Other entry factors

NPC1L1

Besides the above mentioned cellular factors, Niemann-Pick C1-like1 cholesterol absorption receptor (NPC1L1), which is a 13 transmembrane protein located at the canalicular-apical domain of hepatocytes was identified as another additional HCV entry factor (Sainz *et al*., 2012). The NPC1L1 domain specific for HCV entry was mapped to the large extracellular loop1 (LEL1), since antibody-mediated blocking of LEL1 but not LEL2 nor LEL3 significantly reduced HCV infection (Fig.29). The identification of this cholesterol absorption receptor in HCV entry is in line with the important role of cholesterol uptake in HCV entry (Kapadia & Chisari, 2005; Ye, 2007; Bartenschlager *et al*., 2011). More interestingly, the clinically available NPC1L1 antagonist ezetimibe, which is used for reducing blood cholesterol level, was shown to potently inhibit HCV infection in a pan-genotype manner, and also delay the establishment of HCV GT 1b infection in human liver chimeric mice, indicating a new therapeutic option for HCV treatment (Del Campo *et al*., 2012; Lupberger *et al*., 2012).

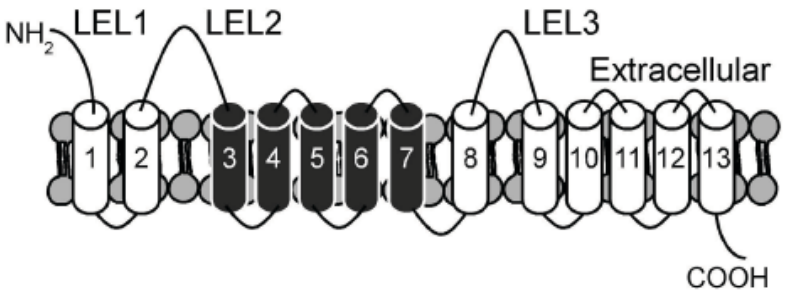


Fig. 29: Membrane topolgy of NPC1L1 (Sainz *et al*., 2012)

TfR1

Coincidentally, the iron uptake receptor, transferrin receptor 1 (TfR1) was also identified as an HCV entry receptor by the same research group (Martin & Uprichard, 2013). Indeed, TfR1 has been described as a ‘‘common’’ viral entry receptor, participating in the entry of three unrelated families of viruses, including a retrovirus, mouse mammary tumor virus (MMTV),

and several rodent and human arenaviruses (Coffin *et al*., 2013). TfR1 was demonstrated to mediate HCV entry dependent on the interaction with HCV envelope glycoproteins. It was shown to participate in HCVpp entry and HCVcc binding to TfR1 expressing CHO cells. Kinetics showed that TfR1 functions at a late step in HCV cell-free entry, after CD81. Also, its role in the HCV cell-cell spread has been investigated by performing foci spread assays. Data showed that TfR1 may enhance HCV cell-cell transmission, but it is not absolutely required for this route (Martin & Uprichard, 2013). The discovery of iron uptake receptor as an HCV entry factor led to a closer look at the link between iron homeostasis and HCV infection. Early studies have reported that patients with chronic HCV infection are often found to have elevated serum iron marker (including ferritin iron and transferrin saturation), and iron accumulation in the liver, which could worsen liver damage (Weiss *et al*., 1994 ; Fabris *et al*., 2001; Bonkovsky *et al*., 2002; Metwally *et al*., 2004). Indeed, it was previously suggested that iron may affect the course of HCV infection through at least three mechanisms including: (i) direct interaction with the cell-mediated immune pathways (Weiss *et al*., 1994; Mencacci *et al*., 1997; Recalcati *et al*., 1998), (ii) causing oxidant stress in nonparenchymal cells, which contributes to the onset of HCV-related liver fibrosis (Pietrangelo *et al*., 2002; Bonkovsky *et al*., 2002) and (iii) providing iron, an essential nutrient of host cell, for the replication cycle of HCV (Kakizaki *et al*., 2000). However, it was found that TfR1 mRNA levels were significantly and consistent down-regulated during HCV infection, which may offer a clue for the blood iron overload associated with chronic HCV infection (Silva *et al*., 2005; Vagu *et al*., 2013).

EGFR and EphA2

A functional RNAs screen helped to identify other host factors for HCV entry, including epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2). Both EGFR and EphA2 are receptor tyrosine kinases (RTKs), exerting important functions in cell biology activities (Schneider & Wolf, 2009). Inhibitors of EGFR and EphA2 (erlotinib and dasatinib) were found to abolish HCV entry and infection in a pan-genotype manner. Further mechanism investigation suggested that RTKs do not modulate HCV binding but facilitate the CD81-CLDN1 association, which could be explained by previous finding that activation of EGFR promotes CLDN1 redistribution (Singh & Harris, 2004; Flores-Benitez *et al*., 2009). The specific domains of EGFR functioning in HCV entry was mapped in both its ligand-binding and kinase-binding domains. Moreover, erlotinib and dasatinib were also shown to efficiently

block HCV cell-cell transmission. Importantly, the anti-HCV effect of erlotinib was confirmed in the uPA-SCID mouse model, further confirming the role of EGFR as an HCV co-receptor for viral entry and dissemination *in vivo*, as well as providing newly possible targets for anti-HCV therapy.

3. The influence of HCV infection on its entry factors

Besides exploiting entry factors for its life cycle, HCV has also been shown to modulate the expression of some of them. As mentioned earlier, HCV infection stimulates LDLr expression to facilitate its proliferation (Syed *et al*., 2014) In contrast, the expression of entry factor TfR1 is down-regulated during HCV infection (Martin & Uprichard, 2013). Recently, HCV replication has also been reported to decrease the cell surface expression of CD81 (Ke & Chen, 2013). Furthermore, the expression of CLDN1 and OCLN were also downregulated during HCV infection. Such downregulated expression following HCV infection was also found to cause a state of resistance to a second infection, suggesting that HCV may achieve the exclusion of superinfection by modulating the expression of its entry factors (Liu *et al*., 2009). However, for the modulation of HCV infection on CLDN1 expression, there seemed to be a controversy. Indeed, in another study, CLDN1 expression was found to be increased on the basolateral membranes of HCV-infected hepatocytes (Reynolds *et al*., 2008). For OCLN, as discussed above, HCV envelope proteins trigger an altered localization of TJ proteins, which mainly concerns OCLN. This mislocalization of OCLN could be due to its retention within the ER by interaction with intracellular envelope protein E2. Interestingly, the altered organization of OCLN and ZO-1 can be partially recovered after IFN-α treatment (Benedicto *et al*., 2008). These findings indicate that HCV infection not only modulates the protein levels of some of its entry factors, but it can also alter their distribution on the surface of infected cells. More recently, an induced endocytosis of CD81 and CLDN1 by HCV particles has also been demonstrated, indicating that HCV stimulates receptor trafficking to facilite its internalization (Farquhar *et al*., 2012).

In summary, HCV infection modulates the expressions and distributions of its entry receptors to facilitate the entry and viral proliferation. Meanwhile HCV infection triggered cellular innate immune responses also induce the expressions of certain ISG proteins on cell surface, where they can interfere with ‘‘normal’’ viral entry process (Schoggins *et al*.,2011; Wilkins *et al*., 2013).

4. HCV entry as antiviral targets for the development of anti-HCV therapy

HCV entry has also been explored for the development of new anti-HCV interventions. Targeting HCV entry could potentially limit viral spread in the HCV-infected livers, but also as a complementary intervention to the ‘‘classic’’ antiviral therapy which is mainly targeting viral RNA replication. The targets of HCV entry can be the viral envelope glycoproteins and also the host factors that are involved in HCV entry.

4.1 Targeting HCV envelope proteins

Monoclonal and Polyclonal neutralizing Immunoglobulins

The presence of neutralizing antibodies in the acute phase of HCV infection were strongly associated with virus clearance (Bowen & Walker, 2005). In addition, the administration of broadly neutralizing antibodies that recognize HCV E2 has been shown to prevent HCV infection in human liver-chimeric mice (Law *et al*., 2008). Furthermore, passive immunization of human liver-chimeric mice with polyclonal immunoglobulins derived from an HCV chronically-infected patient was shown to protect some of these mice from a challenge with homologous and heterologous viruses (Meuleman *et al*., 2005; Vanwolleghem *et al*., 2008; Meuleman *et al*., 2011). However, infection of diverse HCV genotypes via cell-cell transmission route was found to be resistant to anti-E2 neutralizing antibodies and sera from HCV-infected patients *in vitro* (Brimacombe *et al*., 2011). This may explain the observation that some passively-immunized mice failed to be completely protected against HCV challenge.

Interestingly, it has been found that in the plasma of HCV chronic carriers, there exist non-neutralizing/interfering antibodies which recognize the epitope II in E2 (residues 434-446) and interfere with the activity of neutralizing antibodies. Depleting these interfering antibodies from the plasma with epitope-II-specific peptide led to enhanced neutralization (Zhang *et al*., 2009; Tarr *et al*., 2012). However, there is some controversy concerning the existence of these interfering antibodies since some antibodies recognizing epitope II have a neutralizing activity (Sautto *et al*., 2012). Some human mAbs against E1E2 complex such as AR3A, AR3B and AR4A have been preclinically tested in humanized mice (Hadlock *et al*., 2000; Law *et al*., 2008; Giang *et al*., 2012), and other human mAbs recognizing E2 have been

also studied in HCVpp or HCVcc systems (Allander *et al*., 2000; Hadlock *et al*., 2000; Schofield *et al*., 2005; Johansson *et al*., 2007; Owsianka *et al*., 2008; Perotti *et al*., 2008; Broering *et al*., 2009). Antibodies targeting HCV E1 have also been evaluated *in vitro* and *in vivo* as well, and this envelope protein may also be a potential target of anti-HCV therapy (Meunier *et al*., 2008; Verstrepen *et al*., 2011).

Other inhibitors targeting HCV envelope glycoproteins

With the development of the HCVpp and HCVcc systems, a growing number of molecules have been found to inhibit HCV entry. Firstly, both E1 and E2 are heavily glycosylated and the glycosylation sites are highly conserved among different HCV genotypes (Goffard *et al*., 2005; Helle *et al*., 2006; Vieyres *et al*., 2010). Molecules that interact with the N-linked glycans of HCV envelope proteins were shown to inhibit HCV infection through blocking E2-CD81 interaction. For instance, the 11kDa lectin cyanovirin-N (CV-N), which is an aqueous extract from the cyanobacterium Nostoc ellipsosporum, was originally identified as an anti-HIV agent. The mechanism for CV-N-mediated inhibition of HIV infection was demonstrated to be due to its specific interactions with the Man-8 and Man-9 N-linked glycans on HIV envelope proteins, which blocked HIV entry into target cells (Bewley & Otero-Qunitero, 2001; Shenoy *et al*., 2002). Similarly, CV-N was also shown to bind the N-linked glycans of HCV envelope proteins and inhibit HCV entry by blocking their interaction with cell surface receptor CD81 (Helle *et al*., 2006). Another lectin protein, griffithsin (GRFT), which was also originally found to inhibit HIV-1 infection, as well as protect against the infection of severe acute respiratory syndrome (SARS) coronavirus through interacting with N-linked high-mannose oligosaccharides on viral envelope proteins (Ziolkowska *et al*., 2006; Ziolkowska *et al*., 2007), showed a nanomolar range and broad-spectrum antiviral activity against HCV in cell culture (Meuleman *et al*., 2011). In addition to CV-N and GRFT, other carbohydrate-binding agents (CBAs) were also found to selectively inhibit HCV entry at a nanomolar or micromolar level (Bertaux *et al*., 2007). Besides CBAs, other antiviral molecules that inhibit HCV entry through targeting viral particle include the acute-phase protein serum amyloid A (SAA) (Lavie *et al*., 2006; Cai *et al*., 2007), oxidized LDL (von Hahn *et al*., 2006), and the geen tea polyphenol epigallocatechin-3-gallate (EGCG) (Ciesek *et al*., 2011; Calland *et al*., 2012). Other plant extracts such as arbidol (ARB) (Boriskin *et al*., 2006), and silymarin were shown to inhibit not only HCV entry but also several other steps of the HCV life cycle.

In addition to the ‘‘natural molecules’’ inhibiting HCV, the effect of some recombinant peptides against HCV particles were also tested in cell culture. For example, a peptide derived from E2 glycoprotein was shown to inhibit HCV entry at a post-binding step (Liu *et al*., 2010). More interestingly, a peptide derived from HCV NS5A N-terminus exhibited a potent antiviral activity against HCV by targeting the viral particle. This peptide was shown to inactivate both intracellular and extracellular HCV particles (Cheng *et al*., 2008). Consistent with the observation that apolipoprotein apoE is a component of HCV viral particle, a human apoE peptide was shown to potently inhibit both HCVcc and serum-derived viral particles entry into hepatoma cells and PHH by blocking viral binding (Liu *et al*., 2012). This finding suggested a direct role of apoE in mediating HCV-cell binding, but also stressed the potential of developing apoE peptides as HCV entry inhitors.

Furthermore, phosphorothioate nucleotide compounds such as amphipathic DNA polymers (APs) were also shown to inhibit HCV entry, by blocking the postbinding/fusion step. APs displayed a size-dependent and genotype-independent inhibition of HCV infection. Finally, the *in vivo* anti-HCV effect of APs was also tested. It was shown that APs efficiently blocked HCV infection in the uPA-SCID mice (Matsumura *et al*., 2009). The HCVpp system has also been used to screen small molecule libraries which led to the identification of a potent HCV-specific triazine inhibitor, called EI-1, which blocks HCV entry and cell-cell transmission (Baldick *et al*., 2010).

4.2 Targeting host factors involved in HCV entry

Anti-receptor antibodies

As noted previously, cellular factors including SRB1, CD81, CLDN1 and OCLN are the minimal set of receptor combination required for a productive HCV infection. Therefore, developing human antibodies specifically targeting these co-receptors could be very interesting for anti-HCV therapy. As a proof of principle, commercially available anti-CD81 antibodies including JS-81, 1.3.3.22 and 1D5 have been shown to efficiently inhibit HCV infection in cell culture and in liver humanized mice (Molina *et al*, 2008; Scheel *et al*., 2008; Meuleman *et al*., 2008; Gottwein *et al*., 2009). Recently, a novel panel of anti-CD81 monoclonal antibodies were shown to significantly inhibit cell-free infection of diverse HCV genotypes, and particularly an HCV escape variant which was selected during liver

transplantation and re-infecting liver graft. Moreover, among anti-CD81 mAbs that block HCV cell-free entry at a postbinding step, one was also found to inhibit HCV cell-to-cell transmission (Fofana *et al*., 2013).

CLDN1 is another interesting target for the development of anti-receptor monoclonal antibodies. Such anti-CLDN1 antibodies were demonstrated to efficiently inhibit HCV infection of all major genotypes as well as variable HCV quasispecies from HCV infected-patients (Fofana *et al*., 2010). Interestingly, these anti-CLDN1 antibodies are able to impair CLDN1-CD81 association, an essential step in HCV entry, which can lead to new strategies targeting a physiological association between HCV co-receptors (Zona *et al*., 2014).

The entry factor SRB1 is vital not only for cell-free HCV infection, but also plays a dominant role in the cell-cell transmission route. Monoclonal antibodies against SRB1 could be an effective antiviral approach. Antibodies such as C167 and 3D5, which block HDL binding and SRB1 mediated lipid transfer, were shown to display potent inhibitory effects on HCVcc infection (Catanese *et al*., 2007). Moreover, prophylactic administration of a codon-optimized variant of monoclonal antibody C167 protected human liver-chimeric mice from infection with different HCV genotypes (Meulemen *et al*., 2012). Another two anti-SRB1 monoclonal antibodies, mAb8 and mAb151 were shown to efficiently inhibit both cell-free and cell-to-cell spread of HCV infection *in vitro*, as well as prevent HCV infection in humanized uPA-SCID mice. More interestingly, these novel human anti-SRB1 antibodies could block HCV intrahepatic spread and virus replicaton when administrated three days after infection, representing a potential therapeutic tool to prevent HCV reinfection in the allografts after liver transplantation (Lacek *et al*., 2012).

Additionally, specific antibodies against other entry factors including LDLr (Molina *et al*., 2007), EGFR, EphA2 (Luperberger *et al*., 2011) and NPC1L1 (Sainz *et al*., 2012) were also shown to efficiently inhibit HCV entry *in vitro.* However, no antibody against OCLN with an anti-HCV activity has yet been reported. In brief, human monoclonal antibodies targeting these host entry factors, provide a promising future in the anti-HCV therapy, especially for the prevention of HCV reinfection of the liver of transplanted patients.

Small molecules targeting entry factors or their activities

Recombinant soluble peptides targeting host factors have also been developed, and their effect on HCV infection were tested *in vitro*. For instance, a LDLr peptide, r-shLDLR4-292 was shown to inhibit HCV entry by interacting with the lipoprotein component of the virion (Molina *et al*., 2007). Another commercially available recombinant soluble form of LDLr (sLDLR, R&D system) was also shown to specifically neutralize HCVcc infection in a dose-dependent manner (Albecka *et al*., 2012).

In addition to the LDLr peptides, small molecules that block SRB1-mediated HDL uptake, including BLT-2, -3, and -4, were shown to significantly reduce HCVpp and HCVcc infectivity (Nieland *et al*., 2002; Voisset *et al*., 2005; Bartosch *et al*., 2005; Dreux *et al*., 2006). Moreover, small-molecule antagonists of SRB1, ITX-5061 and -7650 which not only block SRB1-mediated lipid transfer but also the interaction of sE2 with SRB1, were shown to prevent HCV infection at a nanomolar range and also potently inhibit neutralizing antibody-resistant cell-cell transmission (Brimacombe *et al*., 2010). Other small molecules which have been clinically approved such as erlotinib (an EGFR inhibitor), dastatinib (an EphA2 inhibitor) and ezetimibe (a NPC1L1 antagonist), in addition to their orignial uses, all were also shown to impair HCV entry *in vitro*. The antiviral activities of these drugs were further evaluated *in vivo*, showing a strong inhibition of HCV infection (Lupberger *et al*., 2011; Sainz *et al*., 2012).

IV. HSPGs and syndecan family

HSPGs are ubiquitously expressed on the surface of nearly all mammalian cells, and are the major component of extracellular matrix. They are also abundant on the surface of hepatocytes and in the matrix of Disse Space (Bishop & Gagneux, 2007; Bishop *et al*., 2007). Proteoglycans are glycoproteins, composed of a core protein branched with one or more glycosaminoglycan (GAG) chains. These GAG chains are linear polysaccharides, consisting of alternately repeating disaccharide units that are variably sulfated. Different disaccharide units assemble different GAG chains, including Hyaluronic Acid (HA), Chondroitin Sulfate (CS), Dermatan Sulfate (DS), Keratan Sulfate (KS) and Heparan Sulfate (HS) (Esko & Selleck, 2002). The alternate units consist of a hexosamine and a uronic acid which are connected by a glycosidic linkage. The types of hexosamine includes D-Glucosamine

($GlcNH_2$) and N-Acetyl-D-galactosamine (GalNAc). The types of uronic acid comprises D-glucuronic acid (GlcA) and L-iduronic acid (IdoA). The hexosamine and the uronic acid are linked by either a type α or a type β glycosidic linkage (Fig.30). The disaccharide unit of HS chains is composed by a Glc A and a $GlcNH_2$ (Lindahl *et al.*, 1998; Esko & Lindahl, 2001; Esko & Lindahl, 2002). One or more HS chains are covalently attached to serine/tyrosine residues of a core protein to form a HS proteoglycan (PG).

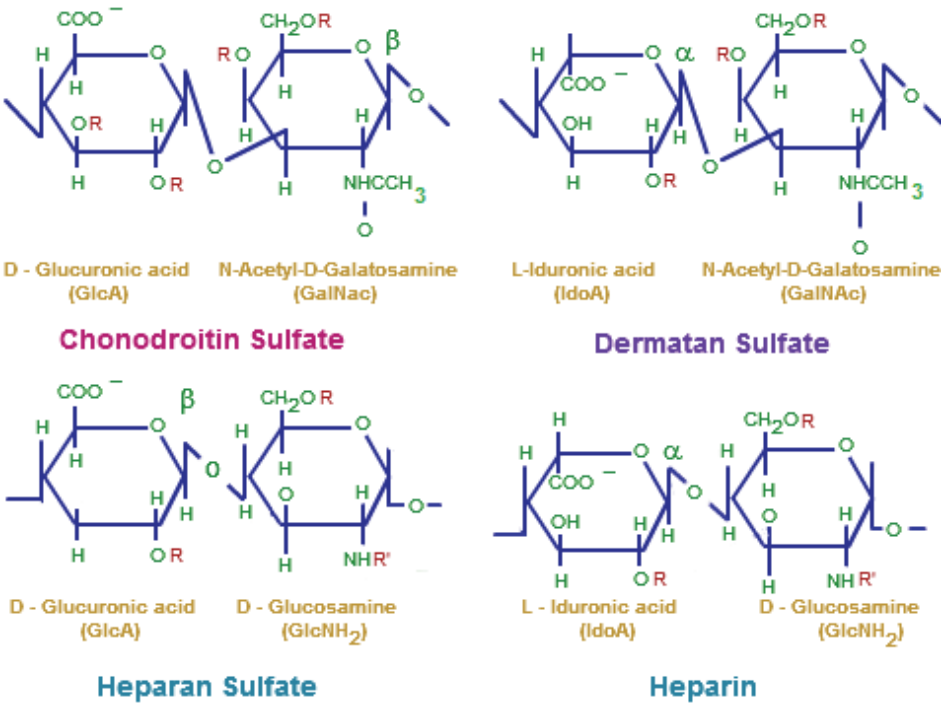


Fig. 30: Molecular structure of different disaccharide units
(source from http://chemistry.tutorvista.com/organic-chemistry/glycosaminoglycans.html)

Based on the PG core protein, cell surface HSPGs are characterized into four major families, including the membrane-spanning syndecans, the glycosylphosphatidylinositol (GPI)-linked glypicans, the membrane-anchored betaglycan and CD44 splice variants. All these HSPG families play distinct roles in cellular adhesion, cell signalling, morphogen gradients and endocytosis (Kirkpatrick & Selleck, 2007). Thus, HSPSs are very important for mammalian development and normal physiology (Bishop *et al.*, 2007).

Table 5. Classification of proteoglycans based on their location and the type of core protein.

Localization	GAG-chain	Mr of the core protein (kD)	Major members
ECM	HA, CS, KS	225-250	aggrecan,versican
Collagen-associated	CS, DS, KS	40	decorin,biglycan fibromodulin
Basement membrane	HS	120	perlecan
Cell-surface	HS, CS	33,60,92	syndecans, glypican, betaglycan, CD44E, cerebroglycan
Intracellular granules	heparin, CS	17-79	serglycin

HSPGs in the basement membranes, interacting with other matrix components, organize the basement membrane barriers and define the barrier functions. For instance, heparan sulfate and syndecan-1 are crucial in maintaining murine and human intestinal epithelial barrier function, and reduced heparan sulfate accumulation in enterocytes could lead to protein-losing enteropathy (PLE) (Bode *et al*., 2008). Heparan sulfate- or syndecan-1-deficient mice showed an increased basal protein leakage and were more susceptible to protein loss induced by increased venous pressure or certain cytokines. A similar pathological phenomenon was also found in syndecan-1 knocking-down human epithelial cells (Bode *et al*., 2005; Bode *et al*., 2006; Bode *et al*., 2008).

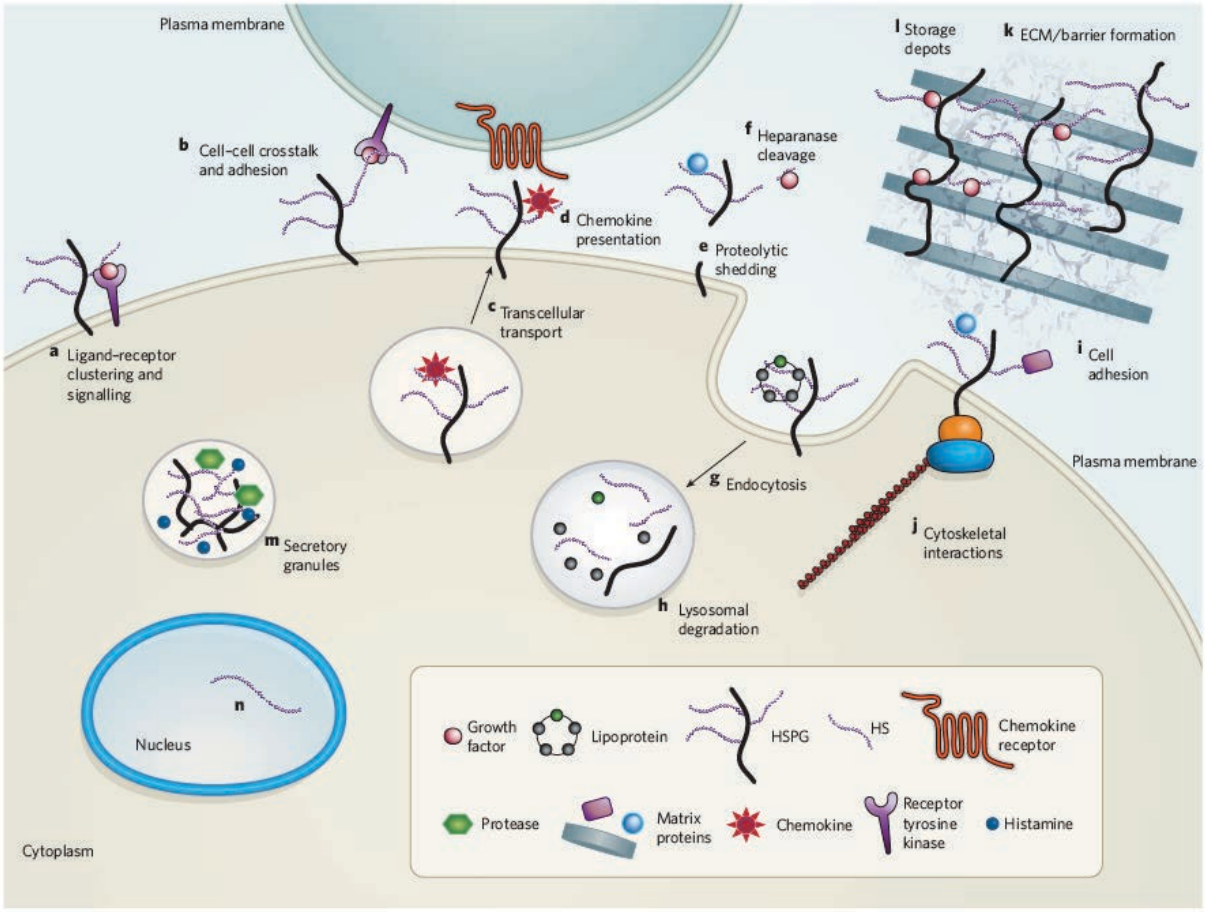


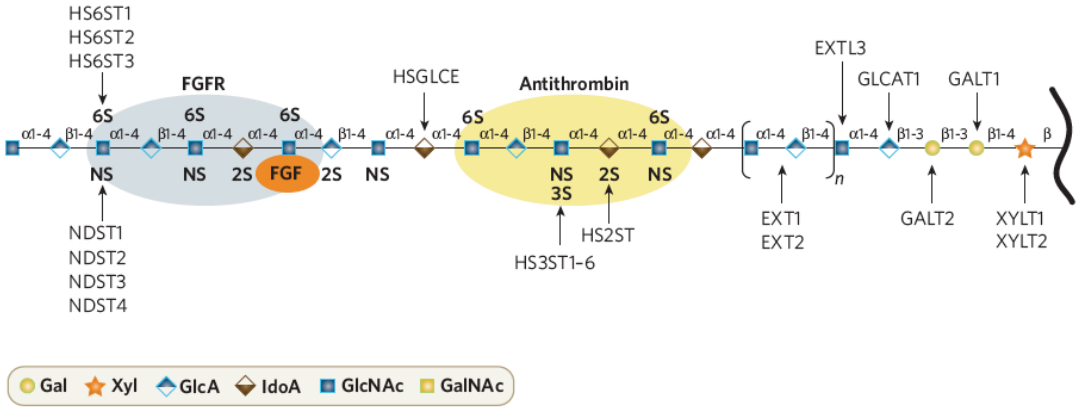
Fig. 31: HSPGs function in variety of cell biological activities (Bishop *et al*., 2007)

(1) HSPGs act as a co-receptor for growth factors and their receptors, which are present on the same cell (**a**), or on the neighbour cell (**b**).
(2) HSPGs transport chemokines across the cytosol (**c**), and present them on the cell surface (**d**).
(3) Proteolytic processing of core protein leads to HSPG shedding (**e**), further cleavage of HS by Heparanase (**f**) liberates the bound ligands (eg. growth factor).
(4) Cell suface HSPGs are actively taken up by cell through endocytosis (**g**), then undergo degradation in lysosomes (**h**) or recycle to cell surface.
(5) HSPGs promote cell adhesion to the extracellular matrix (**i**) and form the bridges to intracellular cytoskeleton (**j**).
(6) Secreted HSPGs organize the formation of extracellular matrix to form physiological barriers (**k**) and sequester molecules including growth factors and morphogens, serving as their storage depots.
(7) Serglycans are packaged into secretory granules of hematopoetic cells (**m**); some HS chains have been reported to be present in the nucleus (**n**), but their function is not clear yet.

HSPGs bind a wide variety of cytokines, chemokines, growth factors and morphogens, protecting them from denaturation and proteolytic degradation, therefore HSPGs serve as a cell-surface reservoir for these molecules. The interactions of HSPGs with morphogen and chemokines facilitate the formation of morphogen gradients and chemokine gradients

(Belenkaya *et al*., 2004; Han & Lin, 2005; Yan & Lin, 2009; Massena *et al*., 2010). The former are essential for cell specialization during developpement (Lin, 2004; Hacker *et al*., 2005), and the latter is involved in leukocyte recruitment and homing, promoting the inflammatory response to infection and injury. Besides being essential for the FGF signaling, HSPGs are also required for other cell signaling molecules such as Wg/Wnts (Tsuda *et al*., 1999), TGF-ß, hepatocyte growth factor (HGF), Hedgehog (Hh) and heparin-binding EGF-like growth factor (HB-EGF) for their optimal signal transductions during Drosophila embryonic development (Zioncheck *et al*., 1995; Reichsman *et al*., 1996; Bellaiche *et al*.,1998; Lin *et al*., 1999; Baeg & Perrimon, 2000; Nybakken & Perrimon, 2002; Lin, 2004).

a.



b.

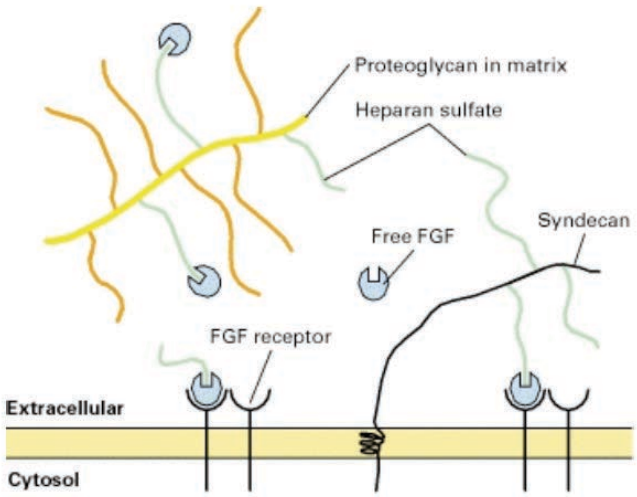


Fig. 32: Heparan Sulfate structure and the binding sites for Ligands (a) and the FGF/HSPG/FGFR ternary complex (b)(Bishop *et al*., 2007)

Importantly, HSPGs modulate the fibroblast growth factor-2 (FGF-2) signaling (Forsten-Williams *et al*., 2005). Indeed, interaction of FGF2 with specific oligosaccharide sequences of HS chains leads to the formation of a FGF2/HSPGs/FGFR1 ternary complex (Fig.32b), inducing the receptor activation and intracellular signaling transduction (Mundhenke *et al.* 2002). This ternary complex formation is required for FGF2 pro-angiogenic activity, hence it is viewed as an appropriate therapeutic target for various angiogenesis-driven diseases including cancer (Jeffers *et al*., 2002; Wesche *et al*., 2011).

Besides FGF2, another well-studied heparin/HS-binding protein is antithrombin, with which heparin interacts to inhibit blood coagulation, functioning as an anticoagulant and an antithrombotic agent used in clinic (Rosenberg, 1975; Lindahl *et al*., 1979; Lindahl *et al*., 1979; Lindahl *et al*., 1984; Petitou *et al*, 2003). Antithrombin is the principal inhibitor of thrombin in plasma, it also inactivates other serine proteinases of the clotting cascade such as Factor Xa and IXa (Olds *et al*., 1994). Heparin binding to antithrombin accelerates the inactivation of thrombin, Factor Xa, and other coagulation factors (Bjork & Lindahl, 1982; Jin *et al*., 1997; Olson *et al*., 2002; Olson *et al*., 2010). Like the interaction between FGF2 and HSPG, the high-affinity binding of antithrombin is also mediated by a specific oligosaccharide sequence on heparin/HS (Fig.32a).The functional form of heparin/HS consists of the pentasaccharide sequence which binds to antithrombin, a hexasaccharide sequence that binds thrombin, and a spacer of four disaccharides between these two specific sequences (Olson *et al*., 1992; Jin *et al*.,1997; Belzar *et al*., 2000). Similar to the role in FGF/FGFR interaction and signaling activation, heparin/HS also serve as a bridge to link antithrombin and thrombin, facilitating their interaction.

1. Syndecans

Among the four HSPG families, syndecan family is the major source of cell surface HSPGs, containing four members (syndecan-1,-2,-3 and -4) (Fig.33). All syndecans are type I transmembrane proteins, consisting of an ectodomain carrying HS and/or chondroitin sulfate GAG chains, a transmembrane domain, and a short cytoplasmic tail which contains the constant and variable regions characteristic of the four members (Tkachenko *et al*., 2005; Bartlett *et al*., 2007). The distinct expression pattern of each syndecan group varies with different or overlapping functions in diverse biological processes and pathological conditions

(Bernfield *et al*., 1999).

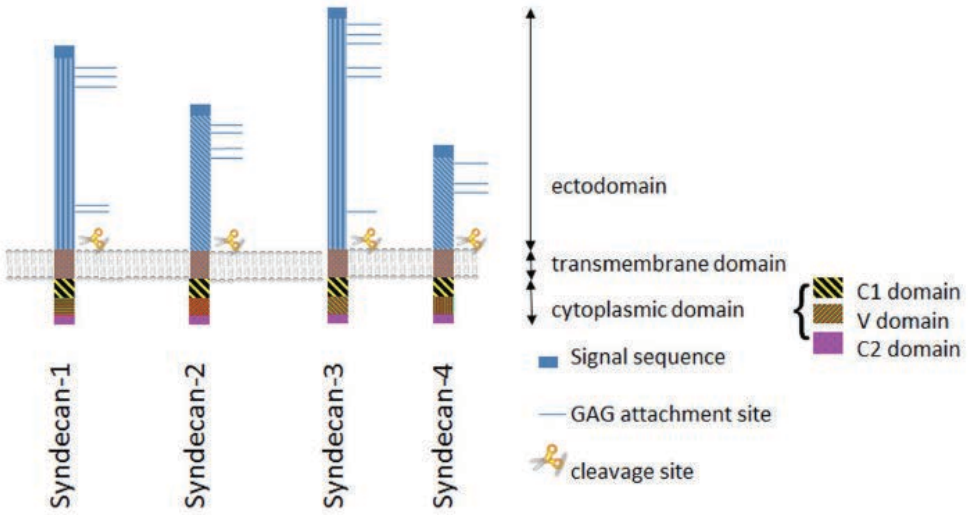


Fig. 33: Schematic illustration of syndecan family (Szatmari & Dobra, 2013)

The extracelluar domains of syndecans are very variable except the attachment sites of GAG chains and the proteolytic cleavage site which is near the plasma membrane. The endo- and trans-membrane domains of syndecans are very conserved (C: conserved, V: variable).

For example, syndecan-1 is present almost exclusively on epithelial cells (Hayashi *et al*., 1987). However, endothelial cells which normally lack syndecan-1 initiate syndecan-1 synthesis in response to wound healing (Elenius *et al*., 1991; Gallo *et al*., 1996). In contrast to syndecan-1, syndecan-2 is not expressed by epithelial cells but mainly produced by endothelial cells and mesenchymal cells-derived fibroblasts (Marynen *et al*., 1989; Lories *et al*., 1992; Gotte, 2003). The epithelial expression of syndecan-1 is consistent with its significance in the regulation of cell-cell and cell-extracellular matrix interactions (Couchman *et al*., 2001). Thus, syndecan-1 is also an important regulator in inflammatory reaction, as enhanced leukocyte-endothelium interaction has been found in the syndecan-1 knock-out mice (Gotte *et al*., 2002; Gotte, 2003). Syndecan-2 is particularly involved in the transforming growth factor-ß (TGF-ß) signaling (Chen *et al*., 2004). Besides their different roles in various biological processes, both syndecan-1 and syndecan-2 have also been found to be crucial for HSV-1 infection (Bacsa *et al*., 2011).

Syndecan-3 has an alternative designation: N-syndecan (neural syndecan), as it was first cloned from rat Schwann cells (Carey *et al*., 1994). Human syndecan-3 is mostly expressed in the nervous system, the adrenal gland and spleen (Berndt *et al*., 2001). Syndecan-3 on

neuronal cells serves as a surface receptor for the heparin binding growth-associated molecule (HB-GAM) which induces the neurite outgrowth of brain neurons (Raule *et al*., 1994; Nolo *et al*., 1995).

Syndecan-4 is ubiquitously expressed but generally at low levels in normal tissues, and its expression level is lower than other co-expressed syndecans in all cell types (David *et al*., 1992; Couchman *et al*., 2003). Interestingly, a switch in syndecan-1 and syndecan-4 expression has been found in Langerhans cells of the epidermis and monocyte-derived DCs during their maturation, which was reported to control cell motility and morphology. In addition, there seemed to exist a functional interconnection between the increased syndecan-4 expression and downregulated syndecan-1 expression, since syndecan-1 downregulation was inhibited by syndecan-4 specific siRNA knock-down (Averbeck *et al*., 2007). Due to their important role in regulating cell adhesion, proliferation and differentiation, syndecans are potentially key regulators of tumor invasion and metastasis. Indeed, syndecan-1 and syndecan-4 were reported to be two independent indicators in breast carcinoma (Lendorf *et al*., 2011).

2. HSPGs in triglyceride-rich lipoprotein (TRL) metabolism

Apolipoproteins as well as some lipases including hepatic lipase, endothelial lipase and lipoprotein lipase have high affinity for heparin or heparan sulfates (Esko & Selleck, 2002). Indeed, by interacting with these lipoproteins and lipases, cell surface HSPGs play important roles in TRL metabolism. The involvement of HSPGs in the lipoprotein metabolism could be considered to begin with their interaction with LPL on surface of the vascular endothelium. After the synthesis in the liver and gut, hepatic VLDL particles and intestinal chylomicrons (CM) are secreted into the plasma. In the circulation, these TRL particles encounter LPL anchored on the endothelial cell surfaces, and undergo the intravascular lipolysis catalyzed by LPL. The physiological function of LPL is to hydrolyze CM and VLDL/IDL particles on the luminal side of capillary endothelium, releasing free fatty acids which are stored as triglycerides (TGs) in adipose tissue or are oxidized for producing energy in the muscle tissues. The intravascular lipolysis removes around 90% of the TGs from TRL particles, yielding remnant lipoproteins which are mainly taken up by hepatocytes (Bergman *et al*., 1971).

The mechanism of remant lipoprotein clearance involves three steps (Mahley & Ji, 1999) (Fig.34). Firstly, plasma lipoprotein remnants are sequestrated into the Space of Disse, where HSGPs ‘‘capture’’ the TRL particles by binding to apoE and hepatic lipases. Of note, apoE secreted by hepatocytes enhances this binding, as secreted apoE binds to TRL particles, enabling them to interact with HSPGs more effectively (Ji *et al*., 1994b; Shimano *et al*., 1994; AI-Haideri *et al*., 1997). In the Disse’s Space, remnant particles undergo further lipolysis by LPL and hepatic lipases (HL), which also serve as ligands for binding to HSPGs, mediating the uptake of TRL particles (Beisiegel *et al*., 1991; Mulder *et al*., 1993; Lauer *et al*., 1994; Shafi *et al*.,1994). Then, cell suface HSGPs, LDLr, LRP and other receptors mediate the endocytosis of the remnant particles by the hepatocytes (Mahley *et al*., 1979; Ji *et al*.,1993; Mahley *et al*., 1994; Herz & Strikland, 2001). In this final step, HSPGs either act alone as a receptor mediating direct uptake of apoE- and HL-containing particles, or HSPGs function together with LRP, internalizing apoE-enriched lipoproteins through the HSPG-LRP pathway (Ji *et al*., 1993; Ji *et al*., 1997; Wilsie & Orlando, 2003) (Fig.34). LDLr, LRP1 and HSPGs are the three major receptors for TRL clearance, since mice lacking hepatic expression of all these receptors exhibited dramatic hyperlipidemia. By analyzing the accumulated remnant particles in mutant mice with single or double receptor deletion, it was found that HSPGs preferentially clear small TRL particles with a 20-40 nm diameter, whereas LDLr and LRP1 mainly remove a set of larger particles (40-60nm in diameter) from the plasma (Foley *et al*., 2013).

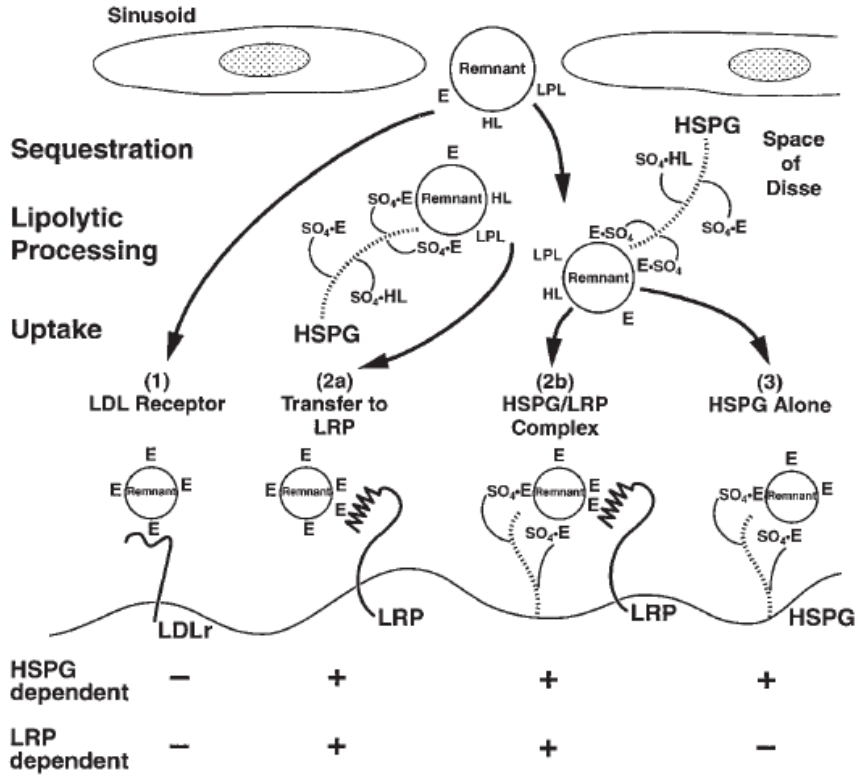


Fig. 34: The mechanism of TRL hepatic clearance and remnants uptake pathways (Mahley *et al*., 2000; Mahley & Rall, 2000)

The clearance of plasma remnant lipoproteins undergoes in three steps: sequestration, lipolytic processing and uptake by the hepatocyte. The major pathways for internalization of remnant lipoproteins are illustrated above: (1) Direct uptake mediated by LDLr, independent of HPSG and LRP; (2) HSPG-LRP pathway (2a) remnant particles are transferred to LRP for uptake, or (2b) remnant particles are internalized through HSPG-LRP complexes; (3) HSPGs alone mediate lipoprotein remnants uptake.

Interestingly, apoE isoforms: apoE2- apoE3-, apoE4-containing lipoprotein particles have similar affinity for HSPGs binding, but the intracellular accumulation/retention of apoE3 or apoE2 was found to be higher than that of apoE4 in the liver cells (HepG2) and other cell types such as Neuro-2a cells and fibroblasts (Ji, *et al*.,1998). The HSPG-dependent/LRP-independent internalization of apoE-enriched particles accounts for the differential intracellular accumulation of apoE3 and apoE4, with the exact mechanisms being unclear. However, it was proposed that apoE3 internalized by HSPG pathway is protected from endosomal degradation, and escapes into the cytoplasm, where it is sequestered (Roses *et al*., 1994; Strittmatter *et al*., 1994). While the HSPG-complexed apoE4 may be recycled to the cell surface and released from the cell (retro-endocytosis), thereby resulting in decreased apoE4 accumulation.

HSPGs bind LPL

The fact that LPL is needed for intravascular lipolysis of circulating TRL has been known for decades (Morley *et al*., 1974; Morley *et al*., 1975; Groot & Van Tol, 1978; Goldberg, 1996) However, the mechanism for LPL binding onto the capillary endothelium remained unknown until recently. Early studies demostrated that LPL is transported to HSPG on the luminal side of endothelium after it is synthesized and secreted from parenchymal cells such as cardiomyocyte, myocyte and adipocyte cells (Cheng *et al*., Shimada *et al*., 1981; Williams *et al*.,1983; Saxena *et al*., 1991). Moreover, it was discovered that endothelial cells-secreted small HS oligosaccharides play an important role in this translocation of LPL. HS oligosaccharides associated with LPL not only protect LPL from inactivation, but also increase the association of LPL with endothelial cells (Parthasarathy & Spiro, 1984). Incubation of endothelial cells with heparin can release cell-bound LPL (Stins *et al*., 1992), and LPL binding can also be reduced by treating cells with heparinases which remove HS chains (Shimada *et al*., 1981). However, the ‘‘HSPG’’ model was perhaps far-fetched, as HSPGs are also expressed on the myocyte and adipotyce cells, it was not clear why LPL would binds to HSPGs on the endothelial cells rather than the HSPGs on the cells where LPL is produced. And another question is how LPL was moved to the capillary endothelium. The discovery of glycosyl phosphatidyl inositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1) shed light into these questions. First, GPIHBP1-knockout mice manifested severe hypertriglyceridemia, indicating the relevance of GPIHBP1 in the lipolytic processing of TRL (Beigneux *et al*., 2007). It was then found that GPIHBP1 is highly expressed in capillaries of heart and brown adipose tissue which produce large amount of LPL, whereas GPIHBP1 is absent from the endothelial cells of arterioles, venules and the capillaries of the brain, an organ using glucose for fuel (Beigneux *et al*., 2007; Davies *et al*., 2010). Further studies revealed that GPIHBP1 binds LPL, and tanslocates LPL from interstitial spaces to the luminal side of capillary endothelium, shuttling across the endothelial cells (Beigneux *et al*., 2009a; Beigneux *et al*., 2009b; Beigneux *et al*., 2010; Davies *et al*., 2010; Goulbourne *et al*., 2012; Goulbourne *et al*., 2014).

HSPGs bind HL

In addition to LPL, two other lipases of the same family, endothelial lipase (EL) and hepatic lipase (HL) also play important roles in the intravascular lipolysis. Like LPL, both EL and HL

bind HSPGs *in vivo* (Krauss *et al*., 1973; Fuki *et al*., 2003; Badellino *et al*., 2006). But, neither EL nor HL binds GPIHBP1 (Beigneux *et al*., 2007; Beigneux *et al*., 2009a; Beigneux *et al*., 2009b). EL is largely generated by the endothelial cells, and its function is to hydrolyze phospholipids in the HDL particles, reducing the size of HDL particles and accelerating their removal from the plasma (Duong *et al*., 2003; Ishida *et al*., 2003; Jin *et al*., 2003). HL is produced by hepatocytes and mainly localized in the sinusoids of the liver (Kuusi *et al*., 1979; Sanan *et al*., 1997; Mahley & Ji,1999). Both secreted and cell surface-bound HL enhance the binding of ß-VLDL/chylomicron remnants (Ji *et al*., 1994). Later study demonstrated that the HL-mediated uptake is indeed mediated by LRP, but initiated by binding to HSPGs (Krapp *et al*., 1996). HL is known to hydrolyze triglycerides and phospholipids. However, its lipolytic activity is neither necessary for HL-mediated enhanced binding nor uptake of remnant particles (Ji *et al*., 1994). Indeed, it has been suggested that HL can function as a ‘‘bridge’’ independent of its enzymatic activity, when both its lipoprotein-binding function and HSPG binding function are intact (Dichek *et al*., 1998). It binds the remnant particles in the Space of Disse, participating in the sequestration step of remnants clearance (Fig.34), then HL-bound particles bind to cell surface HSPG. Therefore, HL serves as a molecular ‘‘bridge’’ bewteen remnant particles and HSPG on cell surface. Similarly, LPL also has this ‘‘bridge’’ function to mediate TRL uptake (Rumsey *et al*., 1992; Mulder *et al*., 1993; Goldberg *et al*., 1996; Seo & St Clair, 1997). As mentioned above, LPL’s bridge function has been reported to play a role in HCV infection. Its action on HCV particles involves the formation of a bridge between HCV-associated lipoproteins and cell surface heparan sulfates (Andreo *et al*., 2007). But, the bridge function led to the immobilization of viral particles at cell surface, thus inhibited HCV infection through blocking virus cell entry (Maillard *et al*., 2011).

The interaction between HSPG /LDLr and ApoE containing lipoproteins

ApoE is known as a critical ligand for the clearance of remnant lipoproteins (Mahley, 1988; Mahley *et al*., 1996; Cooper *et al*., 1997; Mahley & Ji., 1999). Early studies performed in cholesterol-fed rabbits showed that intravenous infusion of apoE decreased plasma cholesterol level by 35-40% within 2-3h, indicating that apoE drove the remnant particles into the liver, accelerating the remnant clearance from plasma. This accelerated clearance presumably reflected the uptake of remnant lipoprotein by hepatocytes, where apoE-containing particles bind to cell surface receptors including LDLr, LRP and HSPGs, mediating the internalization of remnant particles. ApoE has a high affinity for these hepatic

lipoprotein receptors, and defective binding of apoE to these receptors leads to the accumulation of cholesterol-rich lipoprotein particles in the bloodstream, which is also a cause of type III hyperlipoproteinemia (HLP). HLP is also called dysbetalipoproteinemia, characterized by elevated plasma triglyceride and cholesterol levels (Morganroth *et al*., 1975). Type III HLP is a genetic disorder, caused either by apoE deficiency (Mabuchi *et al*., 1989). or expression of apoE forms that are defective in binding to lipoprotein receptors (Rall *et al*., 1982). Some apoE forms are more defective in LDLr binding, while others show more deficiency in the HSPG/LRP binding. For example, apoE2 can not bind LDLr normally, but it can use the backup HSPG/LRP pathway efficiently, thus protecting against apparent hyperlipoproteinemia (Kowal *et al*., 1990; Ji *et al*., 1998). In contrast, expression of apoE mutant form apoE (142Arg➔Cys), which is only partially attenuated in LDLr binding but has a severe deficiency in HSPG binding, resulted in type III HLP in humans with normal LDLr expression (Horie *et al*., 1992). This indicates that LDLr-mediated binding activity alone is not sufficient to mediate normal clearance of apoE-containing remnants. Indeed, type III HLP-associated apoE variants such as apoE142 (Arg ➔Cys), apoE145 (Arg ➔Cys), and apoE146 (Lys➔Glu), all were found to poorly bind HPSG, leading to a severe impairment in the initial sequestration of lipoprotein remnants in the Space of Disse, as well as the subsequent HSPG/LRP binding-mediated remnant clearance (Ji *et al*., 1994). These ApoE variants also displayed a deficiency in LDLr binding (Mahley, 1988). Under normal circumstances, lipoprotein remnants containing apoE can directly interact with LDLr and be uptaken by hepatocytes through the classic LDLr pathway (Browen & Goldstein, 1983; Brown & Goldstein, 1986; Cooper *et al*., 1997). It has been determined that a single apoE molecule binds to LDLr with an affinity similar to that of single LDL molecule (Pitas *et al*., 1980). But, the multi-receptor binding affinities of apoE have an important impact on the hepatic clearance of remnant lipoproteins. When the number of apoE molecule increased to four or more per remnant particle, the total binding events increase markedly through multiple interactions, leading to a corresponding increase in the removal rate of remnant particles (Funahashi *et al*.,1989).

Importantly, the LDLr binding site of apoE overlaps with its heparin-binding site that is the amino acid sequence (aa136-150) located in the N-terminal domain (residues 1-191) of apoE (Wilson *et al*., 1991; Weigraber *et al*., 1994; Dong *et al*., 2001; Libeu *et al*., 2001). The other heparin binding site of apoE is at the C-terminal domain (Cardin *et al*., 1986). However, it is

not available for heparin binding in both lipid-free and lipidated apoE molecules, indicating that only the N-terminal heparin-binding site is involved in the interaction between apoE and HSPG *in vivo* (Weisgraber *et al*., 1986; Libeu *et al*., 2001; Saito *et al*., 2003). It has been well demonstrated that apoE binds to heparin through a two-step process (Futamura *et al*., 2005). In the initial binding step, ApoE binds to heparin with fast association and dissociation rates, which involves the electrostatic interaction with heparin and no major secondary or tertiary structural change is found in apoE. In the following step, it exhibits much slower kinetics with a progressively decreased dissociation rate, which indicates the gradual stabilization of the initial apoE-heparin complexes (Fig.35). In addition to the interaction between apoE and HSPG, this two-step mechanism also provided insights into the mechanism of remnant lipoproteins clearance mediated by HSPG/LRP pathway. As for the first step, apoE-containing remnant particles are captured by the abundant HSPG on cell surface through the fast association of apoE with HSPG, which is a long range and nondirectional ionic interaction, allowing a fast dissociation of the remnant particles from HSPG and a rapid transfer of these particles to LRP that are complexed with HSPG on cell surface (Wilsie & Orlando, 2003). LRP then retains the remnant particles until endocytosis occurs. Alternatively, some initial remnant-HSPG complexes undergo the second binding step, to form a more stable remnant-HSPG complex for internalization (Fig.35).

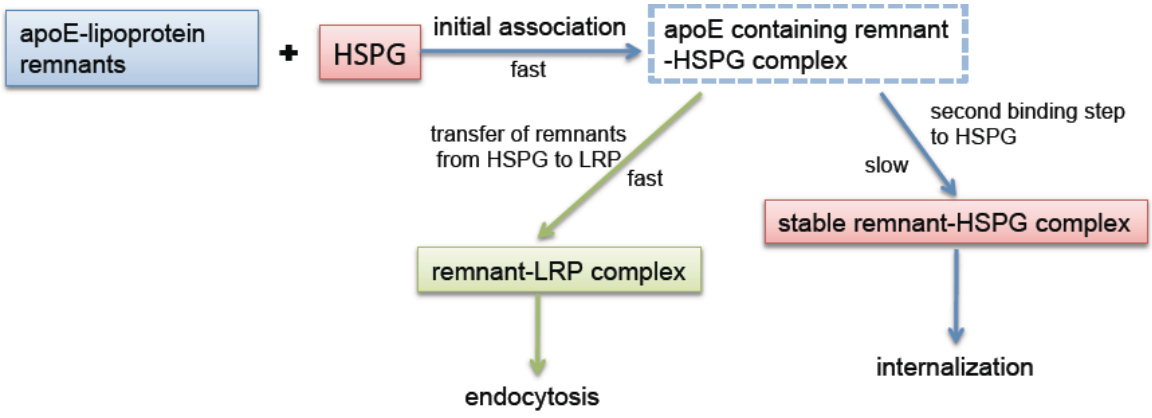


Fig. 35: Two-step mechanism of remnant lipoprotein uptake via HSPG/LRP pathway
Adapted form (Futamura *et al*., 2005).

The apoE-enriched remnant lipoproteins are first captured through the fast association of apoE with cell-surface HSPG. However, because of the fast dissociation rate of this interaction, remnant lipoproteins are rapidly transferred to the LRP to form a stable complex (characterized by slow dissociation) for endocytosis (represented in light green box). Some initial complexes of HSPG-remnant particle would undergo the second binding step, forming a more stable complex for internalization (represented in light red box).

ApoE exists as a self-associated tetramer in the aqueous phase, and it dissociates when it binds to lipid particle surface, remaining as a monomeric form (Funahashi *et al*., 1989). ApoE does not recognize LDLr in the absence of lipids (Yokoyama *et al*., 1990; Innerarity *et al*., 1979). In contrast, lipid association is not required for apoE binding to HSPG (Saito *et al*., 2003; Shuvaev *et al*., 1999) or LRP (Narita *et al*., 2002). Lipid-free apoE can also bind heparin effectively (Mahley *et al*., 1999; Futamura *et al*., 2005). Interestingly, all apoE isoforms have similar affinities for heparin binding (Shuvaev *et al*., 1999), while ApoE binding affinity to LDLr is isoform-specific (Yamamoto *et al*., 2008).

Unlike LDLr binding, effective heparin-binding of apoE does not require charge specificity of basic residues and the amphipathic α-helix in the region 140-150aa (Futamura *et al*., 2005). In turn, the structure requirement for HSPG interacting with apoE was mapped to specific sequences of heparan sulfates (Table. 6). However, it was recently reported that HSPG can also mediate TRL clearance through an apoE-independent pathway, which does not involve apoB but apoA-V (Gonzales *et al*., 2013), another heparin-binding apolipoprotein (Lookene *et al*., 2005).

Table 6. Apolipoproteins and lipases binding to heparin/heparan sulfate

Ligand	Binding specificity	Reference
ApoE	heparan sulfate rich in N- and O-sulfo groups, four repeats of IdoUA(2S)-GlcNS(6S)	Libeu CP *et al*.,2001 JBC
ApoB	a heparin binding region of apoB (apoB10$_{00-10}$ RALVDTLKFVTQAEGAK), N-terminal region of apoB-48,-100	Shih IL*et al*.,1990PNAS, Goldberg IJ 1998JBC, Flood C *et al*.,2002JBC
ApoA-V	binding Heparin, HSPGs	Lookene A2005JBC, Gonzales JC2013JCI
Lipoprotein lipase	(IdceA(2S)alpha1-4GlcNS(6S)alpha1-4) 4-IdceA(2S)AManR(6S)	Pathasarathy N 1994JBC
Endothelial lipase	functioning as a bridging molecule between lipoproteins and HSPGs	Fuki IV2003JBC
Hepatic lipase	binding heparin, HSPGs	JiZS1994JBC, Lee SJ2004JLipidRes

In vivo genetic study identified syndecan-1 as the main HSPG involved in TRL remnant hepatic clearance. Indeed, syndecan-1 deficient mice displayed elevated triglyceride plasma level (Stanford *et al*., 2009). Furthermore, an earlier study has demonstrated that syndecan-1

can mediate the internalization of ^{125}I-apoE -VLDL through a LRP-independent and non-clathrin-mediated pathway (Wilsie *et al*., 2006).

3. HSPGs in microbial infections

In addition to binding to a wide range of ligands and TRL particles, a growing body of studies have shown that heparan sulfate chains of HSPG also provide abundant docking sites to many microbial pathogens such as viruses, bacteria and parasites, facilitating their initial attachment to cell surface and subsequent interactions with specific receptors, prompting their invasion and spread into host cells (Bernfield *et al*., 1999; Rusnati & Urbinati, 2009; Barlett & Park, 2010). Consistent with this mechanism, additionally-added solube HS or its homologue heparin can inhibit HS-binding pathogens binding to cell surface HSPGs, and removing the heparan sulfates by enzymatic treatments or genetic mutagenesis also lead to a reduction in viral infection (Rostand & Esko, 1997). Furthermore, chemically desulfated heparins or heparin oligosaccharides with different length are currently available, which are used to investigate the structural determinants such as the HS modification and the minimal subunits of HS oligosaccharide required for the HSPG-pathogen interactions. CHO cell lines deficient in different HS biosynthetic enzymes have also been used to study the type and degree of HS modification that are important for microbial infections (Shieh *et al*., 1992; Esko, *et al*., 1995; Tuve, *et al*., 2008).

So far, a large number of viruses have been found to subvert HSPGs for their infections (Table. 7). For instance, HSV-1 is one of many viruses that utilize cell surface HS for viral entry (Spear, 2004). Indeed, it is well established that cell surface HS provide the initial docking sites for HSV-1 infection (WuDunn & Spear, 1989; Shieh *et al*., 1992). All viral glycoproteins gB, gC, gD, gH and gL contribute to HSV-1 cell entry (Shukla & Spear, 2001; Spear & Longnecker, 2003; Spear, 2004). Attachment of HSV-1 to cell surface involves the interaction between gB, gC and HS, followed by gD interacting with three specific receptors including herpes virus entry mediator (HVEM), nectin-1 and 3-O-sulfated HS (Fig.36) (Shukla *et al*., 1999; Spear & Longnecker, 2003; Tiwari *et al*., 2005). HSV-1 gB, gD, gH and gL which participate in the virus-cell fusion step are all essential for HSV-1 entry (Spear *et al*., 2000). Similarly, like other HS-binding viruses, HSV-1 infection can be inhibited by heparin or certain forms of HS (Tal-Singer *et al*., 1995). The interaction between viral

glycoprotein gB, gC and HS was proved by the results that both isolated gB and gC bind to heparin or HS directly (Williams & Straus, 1997; Trybala *et al*., 2000). Of particular interest is the observation that 3-O-sulfated HS serves as an HSV-1 entry receptor (Shukla *et al*., 1999). Syndecan-1 has multiple functions in HSV-1 infection. Syndecan-1 and syndecan-2 have been shown to be significant for HSV-1 attachment (Bacsa *et al*., 2011). Importantly, the specific HSPGs syndecan-1 has been demonstrated to play a critical role in HSV-1 induced cell-cell membrane fusion and cell-to-cell transmission, and those functions are independent on the HS chains (Karasneh *et al*., 2001). In addition to its role in HSV-1 infection, syndecan-1 was also shown to support human papillomavirus (HPV) infection (Joyce *et al*., 1999; Giroglou *et al*., 2001), another common human viral infection, which can lead to both benign and malignant epithelial tumors of the skin and mucous membranes (Fernandes *et al*., 2013). HPV is a non-enveloped, dsDNA virus. It has been suggested that the mechanism of syndecan-1 mediated HPV attachment and internalization involves HPV attachment to host cells through the interaction of capisd protein L1 with cell surface syndecan-1, which induces a conformational change in the capsid protein L2, and leads to the activation of L1 and L2-mediated HPV entry (Shafti-Keramat *et al*., 2003; Richards *et al*., 2006).

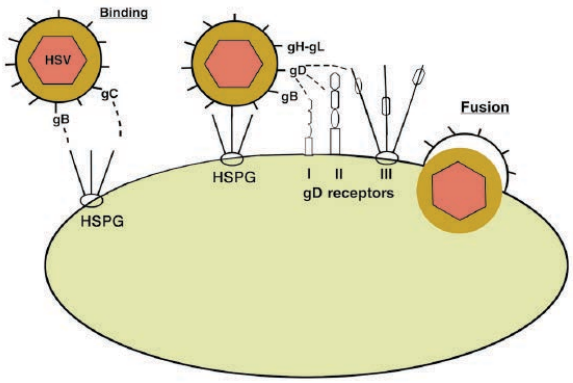


Fig. 36: The mechanism of HSPGs in HSV-1 entry (Shukla & Spear, 2001)

HSV-1 virions attach to target cells through the interaction of viral proteins gB, gC with cell surface HSPGs. Then viral gD protein interacts with specific receptors on cell suface, triggering the fusion of virus with cell membrane. The three receptors for HSV-1 infection are HVEN (I), nectins (nectin-1or nectin-2) (II), and specific 3-O-sulfated HS (III).

Besides HSV-1 infection, HSPGs also play important roles in HIV infection (Morison *et al*., 2001; Kilmarx, 2009; Mumtaz *et al*., 2014). HIV primarily through its glycoprotein gp120 binds to HSPGs on the surface of various cells including epithelial cells, endothelial cells, macrophages, DCs and spermatozoa (Saphire *et al*., 2001; Argyris *et al*., 2003; Bobardt *et al*., 2003; de Witte *et al*., 2007; Ceballos *et al*., 2009; Smith *et al*., 2010). Specifically, HSPG

syndecan-3 was found to be the major HIV-1 attachment factor on DC cells, participating in HIV-1 trans infection (Bobardt *et al*., 2003; de Witte *et al*., 2007). Furthermore, syndecan-1 is upregulated in HIV-infected patients and it is associated with viral translocation of HIV-1 across the gut epithelium (Smith *et al*., 2010). Moreover, HIV-1 Tat protein (Trans-activating regulatory protein) can be released from infected cells and enters neighboring cells through cell surface HSPGs (Franke & Pabo, 1988; Tyagi *et al*, 2011). And, Tat homodimers can bind to HSPGs expressed on lymphoid cells and endothelial cells stimultaneously, forming HSPG/Tat-Tat/HSPG quaternary complexes that link lymphoid cells to the endothelium, providing an alternative mechanism of lymphocyte extravasation in HIV-infected patients (Urbinati *et al*., 2009).

HCV as well as other viruses from *Flaviviridae* family including Yellow Fever Virus (Nickells *et al*., 2008), West Nile Virus, Dengue Virus (Chen *et al*., 1997; Hilgard & Stockert, 2000) and Japanese Encephalitis Virus (Lee *et al*., 2004) (Table. 7), have been reported to use HSPGs for viral infection. As already mentioned at the begining of this part, cell surface HSPGs have been found to be the attachment factor for HCV infection (Germi *et al*., 2002; Morikawa *et al*., 2007). However, the importance of HSPG core proteins in HCV attachment was not clear. Until recently, Luo group specifically silenced the expression of HSPG core proteins through siRNA-mediated knockdown to profile 12 HSPG core proteins in HCV attachment and infection (Shi *et al*., 2013). They found that among these core proteins, only the silencing of syndecan-1 led to an obvious inhibition in HCV attachment and infection, suggesting that syndecan-1 serves as the major receptor for HCV attachment to the surface of hepatocytes. However, these data were challenged by another study that identified syndecan-4 as the major attachemnt factor for HCV (Lefévre *et al*., 2014). More importantly, the interaction between HCV and HSPG has been suggested to be mediated by viral component apoE by the same group (Jiang *et al*., 2012). In their study, an anti-apoE antibody but not HCV E2-sepcific antibody efficiently inhibited HCV attachment, and substitutions of positively charged amino acids with neutral or negatively charged residues in apoE receptor-binding region dramatically blocked HCV attachment. Also, an apoE peptide efficiently inhibited viral binding. These findings seemed to be controversial to the findings from early studies, in which the authors suggested that HCV cellular binding required HCV envelope protein E2 interacting with HSPG, particularly the HVR1 of E2 was proposed to be responsable for HCV-HSPG interaction (Barteh *et al*., 2003; Barth *et al*., 2006). Consistent with this model, it was recently shown that HVR1-deleted mutant virus exhibited a heparin-

resistant phenotype (Koutsoudakis *et al*., 2012).

A variety of bacteria such as staphylococcus aureus (Hess *et al*., 2006; Hayashida *et al*., 2010), streptococcus pneumoniae (Chen *et al*., 2007), pseudomonas aeruginosa (Park *et al*., 2000) and bacillus anthracis (Popova *et al*., 2006) have been reported to utilize syndecan-1 for their infections. Moreover, these bacteria secrete virulence factors that induce syndecan-1 shedding of host cell, and subvert the soluble syndecan-1 ectodomains to inhibit host immune defenses (Park *et al*., 2001; Park *et al*., 2004) (Fig.37). Indeed, pathogen-induced protein ectodomain shedding is not restricted to syndecan-1, since a variety of inflammatory mediators were found to be induced to shed from host cell surface by several pathogens (Vollmer *et al*., 1996; Walev *et al*., 1996; Schmidtchen *et al*., 2001; Lemjabbar & Basbaum, 2002). Moreover, shedding of syndecan-1 was demonstrated to play an important role in the pathogenesis of pseudomonas aeruginosa, contributing the bacterial sepsis caused by p. aeruginosa infection (Hayne *et al*., 2005). Also, it was found that purified ectodomains but not syndecan-1 can counteract neutrophil-mediated killing of staphylococcus aureus, promoting the pathogenic process in the cornea (Hayashida *et al*., 2010). Besides the role of syndecan-1 ectodomain in the pathogenesis of various pathogens, syndecan-1 cytoplasmic domain was also found to be directly involved in the microbial pathogenesis. For instance in Neisseria gonorrhoeae infection, overexpression of syndecan-1 mutants lacking the intracellular domain in epithial cells, lead to an abrogated uptake of the bacteria, suggesting that syndecan-1 intracellular domain plays a crucial role in the Neisseria gonorrhoeae invasion process probably through mediating signal transduction (Zimmermann & David, 1999; Meyer *et al*., 2000).

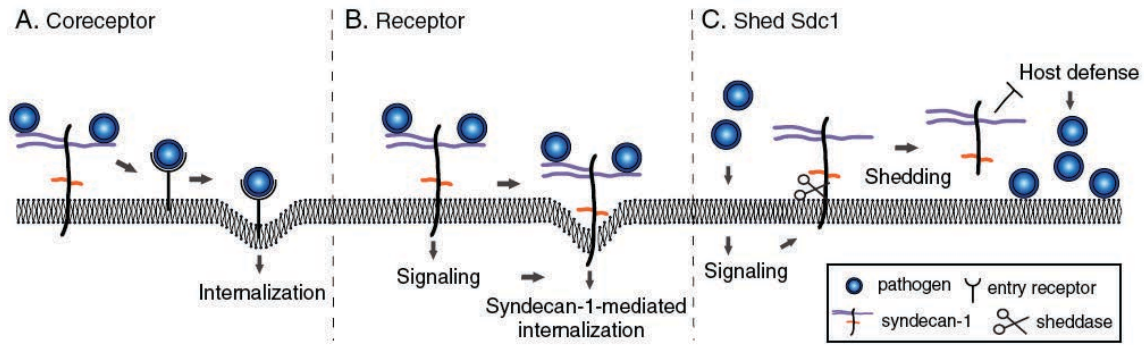


Fig. 37: Mechanisms of syndecan-1 in viral and bacterial infections (Teng *et al*., 2012)

A. Syndecan-1 is subverted as a co-receptor that localize and concentrate pathogens on cell surface and facilitates their interactions with specific receptors. eg: HSV-1

B. Certain pathogens can use syndecan-1 as a direct entry receptor, the pathogen-syndecan-1 interaction stimulates intracellular signaling, leading to syndecan-1 mediated internalization.
eg. N.gonorrhoeae (Freissler *et al*.,2000).

C. Some bacterial pathogens induce syndecan-1 shedding to exploit the ectodomains of syndecan-1 for interfering host immune response. eg. P.aeruginosa (Bucior *et al*., 2010), P.gingivalis (Andrian *et al*., 2005; Andrian *et al*., 2006).

Table 7. HSPG in a variety of viral infections

Viruses	Viral protein	HSPG	Function/interaction	Reference
Adeno-associated virus type-2	Capsid protein VP3	_	Attachment	Opie 2003
Adenovirus	Ad3 Fiber knob	_	Attachment	Tuve 2008
Coronavirus	Spike protein	_	Attachment	de Haan 2008
Coxsackievirus	Capsid protein VP1	N-, and 6-O-s-ulfated HSPGs	Attachment, endocytosis	Zautner 2003, 2006
Cytomegalovirus	gB	_	Attachment	Boyle and Compton,1998
Dengue virus	envelope protein	_	Attachment, internalization	Chen 1997, Hilgard and Stockert 2000
FMDV	VP3	_	Attachment	Fry 1999
HSV-1,HSV-2	gB, gC, gD	Syndecan-2	Attachment	Spear 2004, Cheshenko 2007
HBV	L envelope protein	_	Attachment	Schulze 2007
HCV	E2	HS, syndecan-1	Attachment	Bath 2006 , Qing2013
HHV-8(KSHV)	gB, gpK8.1A	_	Attachment	Akula 2001,Veeltil 2006
HIV-1	Tat protein gp120 gp41	Perlecan Syndecan-3 Agrin	Tat internalization Attachment Attachment	Argyris 2003,Urbinati 2009 de Witte 2007 Alfsen 2005
HPV	L1 carboxy terminal	Syndecan-1,-3, -4, glypican-1	Attachment	Joyce 199 Shafti-Keramat 2003 de Witte 2008
HTLV1	gp46	_	Attachment	Pinon 2003
JEV	envelope protein	_	Attachment	Lee 2004
Pseudorabies virus	Glycoprotein C	_	Attachment	Trybala 1998
RSV	Fusion glycoprotein	_	Attachment	Crim 2007
Rhinovirus	VP1	_	Attachment	Vlasak 2005
Sindbis virus	E2	_	Attachment	Ryman 2007
Vaccinia virus	envelope protein A27L	_	Fusion	Hsiao 1998
WNV	E	_	Attachment	Lee 2004
Yellow fever virus	E	_	Attachment	Nickells 2008

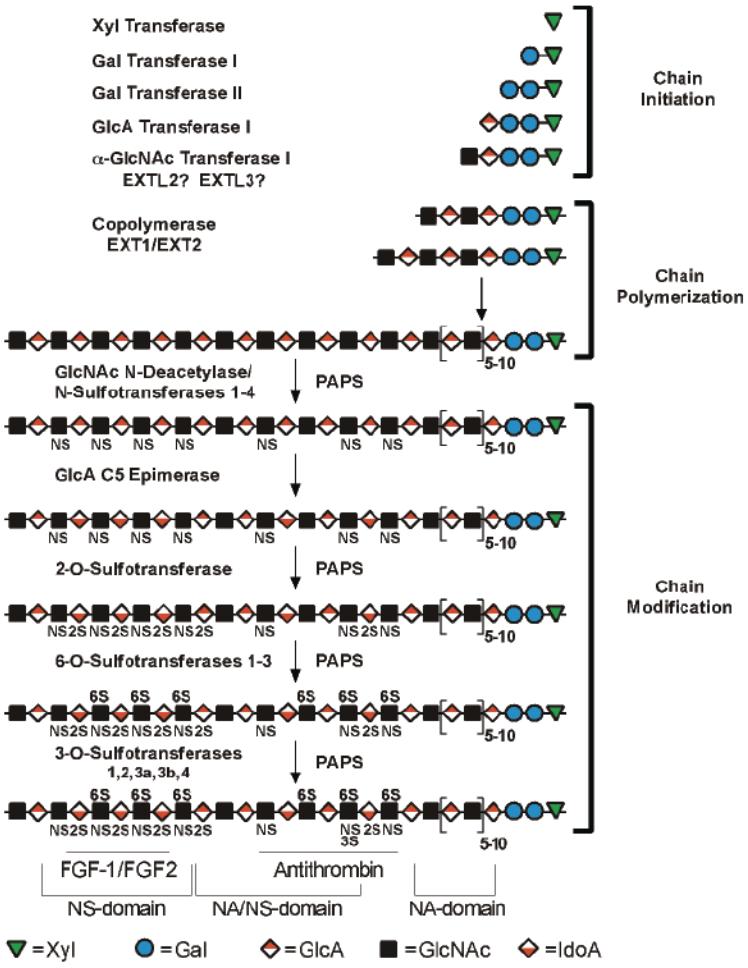
Adapted from (Bartlett & Park, 2010).

4. Biosynthesis of heparan sulfate (HS) chains

HS probably is the most complex polysaccharide in animals. The mechanism of HS biosynthesis has not been completely elucidated. It is generally divided into three main steps including chain initiation, chain polymerization and chain modification (Sarrazin *et al*., 2011) (Fig.38). HS biosynthesis is initiated by the attachment of xylose to specific serine residues in HSPG core protein (Kim *et al*., 2003), followed by the formation of a tetrasaccharide linkage consisting of glucuronic acid-galactose-galactose-xylose (GlcA-Gal-Gal-Xyl) (Lidholt & Lindahl, 1992). After the first N-acetyl-D-glucosamine (GlcNAc) residue is attached to the tetrasaccharide linkage, an enzyme complex Ext1/Ext2 alternately adds the GlcA and GlcNAc residues to the growing polymer, composed by repeating ß 1, 4-GlcA and α 1, 4-GlcNAc disaccharide subunits (McCormick et al., 2000; Senay et al., 2000; Zak et al., 2002). Simultaneously, this poly-oligosaccharide chain undergoes a series of modification reactions mediated by a number of specific enzymes or enzyme families. The primary modification is catalyzed by the bifunctional enzymes N-deacetylase/N-sulfotransferases (NDSTs), which remove the acetyl group from GlcNAc residues, and substitutes it with sulfate (Wei *et al*., 1993; Aikawa & Esko, 1999; Carlsson *et al*., 2008). This N-sulfation reaction is regarded as the key step for HS chain modification, as it is obligatory for the following modification reactions (Bame & Esko, 1989; Bame *et al*., 1991; Bame *et al*., 1994). After the N-sulfation, epimerase GlA C5 epimerizes the D-glucuronic acids that are near the N-sulfoglucosamine units (N-sulfated GlcAc) to iduronic acids (IdoA) (Feyerabend *et al*., 2006; Properzi *et al*., 2008). Then a series of O-sulfo-transferases can add sulfo groups to corresponding positions on the IdoA or GlcAc residues. Specifically, 2-O-sulfo-transferase (2OST) adds sulfate at the C2 position of IdoA (Rong *et al*., 2001). The 6-O-transferases (6OST-1,-2,-3) mainly add sulfate to the C6 position of N-sulfated GlcAc residues, less frequently to the unmodifed GlcNAc (N-acetylated) residues. 3-O-transferases (3-OST-1-7) add sulfate at the C3 site of glucosamine units either N-sulfated or N-acetylated (Fig.38b). Notably, these enzymes do not consistently modify HS chain, since modification reactions occur in clusters of variable length rather than along the full length of HS chain. Domains rich in sulfates (NS domains) are interspersed by unmodified domains (NA domains) (Fig.38a). The junction region between NS domain and NA domain is called NA/NS domain where there exists less extent modification. Eventually, these differentially modified domains constitute multiple binding sites for HS-binding protein ligands such as FGF, Antithrombin, viral proteins (eg. gB, gC

and gD of HSV-1 and HIV gp120), and apoE-containing lipoprotein particles. In other words, the binding sites for these ligands and viral proteins actually are a small part of HS chains.

a.



b.

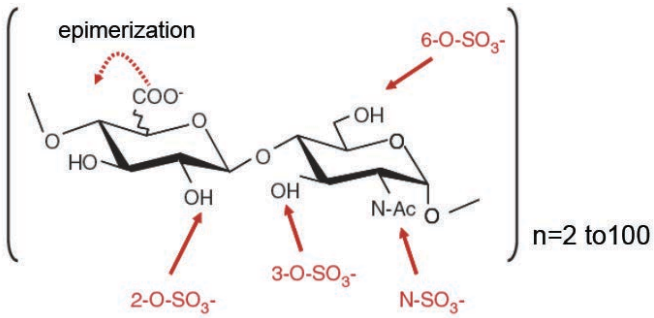


Fig. 38: Schematic drawing of the HS biosynthetic pathway (a) and the sites of sulfations modification (b) (Esko & Selleck, 2002; Connell & Lortat-Jacob, 2013)

The biosynthesis of HS mainly takes place in the Golgi apparatus (Lindahl *et al*., 1998; Esko & Lindahl, 2001; Multhaupt & Couchman, 2012), but when they arrive at cell surface or when they are exposed in the ECM, synthesized HS chains can be further modified by two 6-O endosulfatases Sulf-1 and Sulf-2, which specifically remove the sulfo groups located at the C6 of N-sulfated GlcAc units (Dhoot *et al*., 2001; Ai *et al*., 2003; Hossain *et al*., 2012; Nagamine *et al*., 2013). Furthermore, HS chains can be cleaved by extracellular heparanases, which specifically cleave the linkage between a GlcA unit and an N-sulfo glucosamine unit carrying either a 3-O-sulfated or a 6-O-sulfated group (Peterson & Liu, 2010).

It is very important to point out that there is no single HS or heparin structure. HS biosynthesis is dynamic and tightly regulated, depending on the tissue and developmental stage (Esko & Selleck, 2002; Rudd & Yates, 2012). HS from various mammalian tissues revealed tissue-specific differences in the composition, overall sulfation and domain organization (Lyon *et al*., 1994a; Lyon *et al*., 1994b; Lindahl *et al*., 1995; Maccarana *et al*., 1996; Ledin *et al*., 2004; Kreuger *et al*., 2006; Warda *et al*., 2006; Lindahl & Li, 2009). Human liver is known to be an organ rich in heparan sulfates (Lyon *et al*.,1994a; Vongchan *et al*., 2005), and the specific highly sulfated sequences of HS chains in the liver implicate their important roles in the hepatic clearance of apoE-containing TRL particles (Stanford *et al*., 2010), as well as in infections with hepatotropic pathogens such as malaria sporozoite, dengue virus and HCV (Lin *et al*., 2002; Pradel *et al*., 2002; Barth *et al*., 2003).

The dominant factor for HS-ligand binding is the structural variability of protein-binding sites within maturing HS chains, which is largely contributed by differential expression patterns of HS biosynthetic enzymes (Shworak *et al*., 1993; Chen & Lander, 2001; Tveit *et al*., 2005). Totally, there are around 26 enzymes participating in the biosynthesis of HS chains (Sarrazin *et al*., 2011). Selective modification at different positions, together with the number of modifications lead to the extensive sequence diversity in HS chains (Rosenberg *et al*., 1997; Esko & Lindahl, 2001; Kreuger & Kjellen, 2012). These sulfation modifications are essential for the ligand binding of HS chains. Moreover, NDSTs-catalyzed N-sulfation is specific to HS chains, since CS or DS chains do not contain N-sulfations but O-sulfations (Kjellen *et al*., 2003; Kusche-Gullberg & Kjellen, 2003). Among all sulfotransferases, the 3-O-transferases comprise the largest sulfotransferase family, including seven members, but the 3OST-catalyzed 3-O-sulfation occurs quite rarely. Furthermore, different 3OST isoforms can

produce different ligand-binding sequences on the HS chains (Shworak *et al*., 1997; Xia *et al*., 2002; Xu *et al*., 2005; Lawrence *et al*., 2007). For example, 3-OST1 generates the antithrombin-binding sequence (Lindahl *et al*., 1980), while 3-OST3A produced sequence is structurally different from the antithrombin binding site and is required for HSV-1 gD binding, though its normal biological function is unknown (Shukla *et al.*, 1999; O'Donnell *et al*., 2006; O'Donnell & Shukla, 2009). Interestingly, the isoform 3OST-5 can generate both these binding sites (Xia *et al*., 2002).

The 3OST enzymes compose the largest family of HS sulfation enzymes and the 3OST-modified HS has been proposed to be important in regulating some biological processes (Kreuger *et al*., 2001; Lawrence *et al*., 2007), however most known ligands do not need 3-O-sulfation of HS chains, such as for instance FGF1/2 (Guimond *et al*., 1993; Kreuger *et al*., 2001) and LPL (Parthasarathy *et al*., 1994; Spillmann *et al*., 2006). These ligands binding to HS chains require either the N-sulfation and 2-O-sulfation or 6-O-sulfation. Particularly, the 2-O-sulfation of HS is required for the interactions of many growth factor with their receptors, playing important roles in cellular functions and embryon development (Ornitz, 2000; Kreuger *et al*., 2001; Wilson *et al*., 2002; Kreuger *et al*., 2005). However, the formation of complex between FGF1 and FGF receptor FGF1/HS/FGFR do not require apparent specificity of HS oligosaccharide chains, as FGF1 and its receptor associate at the sites of variable oligosaccharide sequences with different sulfate contents (Kreuger *et al*., 2001). But the lack of both 2-OST and 6-OSTs enzymes can lead to impaired FGF signaling, suggesting a compensatory mechanism between 2-OST and 6-OSTs. This was supported by the evidence that inactivation of 2OST results in enhanced 6OSTs-mediated sulfations, and vice versa (Merry & Gallagher, 2002; Kamimura *et al*., 2006; Dejima *et al*., 2013). However, mice deficient of 2OST suffer from renal agenesis, with other organs developed normally, suggesting that different functional specificities between 2-OST and 6OSTs do exist (Bullock *et al*., 1998). And it has been demonstrated that the 2-O-sulfation but not 6-O-sulfation plays a key role in the lipoprotein binding of HS chains (Stanford *et al*., 2010).

Since NDSTs and 2-, 3-, 6-OSTs add sulfate groups to specfic sites in the HS chains to form the overall sulfation patterns, any abnormality in the expression of these enzymes could lead to altered sulfation patterns. For example, separately overexpression of different isoforms of NDST resulted in the level of N-sulfation from 40% in control cells to 60% in NDST1-

transfected cells, and to 80% in NDST2-transfected cells (Pikas *et al*., 2000). Still in human kidney 293 cells, overexpression of 6OSTs (6OST-1,-2 -3) did not give rise to differently sulfated domains, but increased the 6-O-sulfation on both N-sulfated and N-acetylated GlcAc units (Do *et al*., 2006). In theory the 6-O-sulfation reaction requires the forward N-sulfation, however, inactivation of the enzyme NDST1 in the hepatocytes led to a significantly reduced 2-O-sulfation but had less effect on 6-O-sulfation (MacArthur *et al*., 2007).

5. Heparin and heparin oligosaccharides

Heparin is highly similar to HS in structure, but there are still some distinguishing features between the two. Firstly, heparin is found in the granules of mast cells from connective tissues, while HS are ubiquitously distributed on the cell surface and in the ECM (Humpries *et al*., 1999; Nader *et al*., 1999). Secondly, HS are attached to different core proteins to form HSPGs, whereas heparin is bound to serglycin core protein (Kejellen & Lindahl, 1991; David et al., 1993; Iozzo *et al*., 1994). Finally, they also differ in the degree of sulfation, with heparin being higher N- and O-sulfated (Gallagher & Walker, 1985). HS are 30-60% N-sulfated with a total sulfate (N- and O-) content around one residue per disaccharide unit, while heparin is 80%-90% N-sulfated with the total sulfate content of approximately two residues per disaccharide unit. HS are chracterized by highly sulfated domains (NS) being interspersed with nonsulfated domains (NA) or mixed (NA/NS) domains (Maccarana *et al*., 2006), whereas heparin is uniformly sulfated and resembles more a continuous NS domain of HS.

Table 8. Differences between Heparan sulfate and Heparin

	Heparan Sulfate	**Heparin**
size	10-70 kDa	10-12 kDa
sulfate/disaccharide unit	0.8-1.8	1.8-2.4
GluN N-sulfates	40-60 %	>80 %
IdoA content	30-50 %	>70 %
Binding to antithrombin	0-0.3 %	~30%
site of synthesis	Virtually all cells	Mast cells

Commercially, heparin is isolated form pig intestinal mucosa or bovine lung, and is widely used as an important anticoagulant drug in the clinic. Indeed, it is the 3-O-sulfated pentasaccharide sequence of heparin/HS that binds antithrombin III (Lindahl *et al*., 1980; Ragazzi et al., 1987; Jin *et al*., 1997), relevant to the therapeutic importance of heparin. With a number of heparin-binding proteins being identified, many heparin/HS oligosaccharide sequences required for the HS-protein interactions have been investigated. For instance, the authentic saccharide motifs required for FGF1 binding are octasaccharides containing a highly sulfated trisaccharide motif IdoUA (2-OSO3)-GlcNSO3-(6-OSO3)-IdoUA(2-OSO3), whereas FGF2 binds to hexasaccharides carring a mono-sulfated unit IdoUA(2-OSO3) (Maccarana *et al*., 1993; Tyrrell *et al*., 1993; Faham *et al*., 1996; Kreuger *et al*., 2001 ; Jemth *et al*., 2002; Delehedde *et al*., 2002). A tetrasaccharide size was found to be sufficient for FGF1 and FGF2 binding, but for the biological activity heparin/HS octasaccharide or longer size is required (Delehede *et al*., 2002). Investigation using an octasaccharide library comprising 2-O-sulfated and/or 6-O-sulfated octasaccharides demonstrated that heparin-binding growth factors can be classified roughly into five different groups based on the binding affinity to these octasaccharides (Ashikari-Hadaa, 2004). Apparent discrepancies among different FGF/HS/FGFR interactions have been found in regard to the structure and minimal size of heparin/HS required for binding growth factors and stimulating cell signalling (Ostrosky *et al*., 2002; Mohammadi *et al*., 2005).

The significance of identifying the minimal oligosaccharide motifs within the heparin/HS required for specific protein interactions lies in the enhancement for the specificity of the therapeutic intervention (Rek *et al*., 2009; Gesslbauer *et al*., 2013). For example, the pharmacologically active heparin indeed can cause severe side effects in the clinic including hemorrhage, thrombocytopenia, osteoporosis and increased potassium levels (Hirsh & Raschke, 2004; Hirsh *et al*., 2005; Tang *et al*., 2005). For this reason, low molecular weight fractions of heparin (LMWH) have been developped, which could retain the pentasaccharide binding site for antithrombin III, but have limited interactions with other ligand proteins (Linhardt & Gunay, 1999; Chen *et al*., 2005). For instance, the synthetic pentasaccharides ‘‘Fondaparinux’’ bind rapidly and strongly to antithrombin but not to platelets, indicating its promising clinical applications (Turpie *et al*., 2002; Bauer *et al*., 2002; Bauer et al., 2003; Tran & Lee, 2003; Turpie *et al*., 2003; Nijkeuter & Huisman, 2004; Petitou & van Boeckel, 2004). Besides the inhibition of blood coagulation, heparin also has several ‘‘non-

anticoagulant'' functions such as anti-allergy, anti-inflammation and immunoregulatory (Nelson *et al*., 1993). This is due to the interactions of heparin with various growth factors, proteases and enzymes, which are involved in the modulation of cellular proliferation and angiogenesis, implicating the potential applications of heparin and its oligosaccharide derivatives in inflammation and cancer (Matzner *et al*., 1984 ; Castellot *et al*., 1989; Tyrrell *et al*., 1999; Hochart *et al*., 2006; Yip *et al*., 2006; Casu *et al*., 2010). Importantly, many biological functions of heparin including its anticoagulant and anti-allergic activities were found to be molecular-weight dependent (Cifonelli *et al*., 1974; Laurent *et al*., 1978; Campo *et al*., 1999). Recently, a synthetic heparin-derived pentasulfated (without 3-O-sulfation) tetrasaccharide was shown to have potent effects on the allergic airway response in the antigen-induced early and late airway responses (EAR and LAR), as well as the hyperresponsiveness (AHR) in the allergic sheep and mouse models (Ahmed *et al*., 2012). As already mentioned above, apoE, in particular the isoform apoE4 is genetically linked to AD, and the synthetic apoE peptide or the N-terminal fragment of apoE or full-length apoE were demonstrated to have neurotoxic effects *in vitro*, in an isoform-specific manner (Crutcher *et al*., 1994; Jordan *et al*., 1998; Michikawa & Yanagisawa, 1998; Hashimoto *et al*., 2000). Hopefully, this neurotoxicity can be inhibited by heparin oligosaccharide containing eight or more saccharide residues with four N-sulfo groups and seven O-sulfo groups (Dong *et al*., 2001; Bazin *et al*., 2002).

Owing to the involvement of HS in viral infections, a number of HS mimetic compounds including heparin and heparin-derived oligosaccharides have also been extensively evaluated for their antiviral activities. Heparin has been shown to block dengue-2 virus infection in five human liver cell lines (Germi *et al*., 2002; Lin *et al*., 2002). More interestingly, a mimetic peptide of HIV-1 receptor CD4 was covalently linked to a HS deca-disaccharide to form a bivalent molecule (mCD4-HS_{12}). It can simultaneously bind to CD4, cell surface HS and the co-receptor binding site of HIV-1 gp120, blocking the interactions of HIV-1 with its major cell surface receptors/coreceptors. This HS mimetic compound efficiently inhibits viral infection by blocking HIV-1 attachment and entry (Baleux *et al*., 2009; Tilton & Doms, 2010; Connell & Lortat-Jacob, 2013). Also, a synthetic 3-O-sulfated octasaccharide has been shown to inhibit HSV-1 infection in a dose-dependent manner (Hu *et al*., 2011).

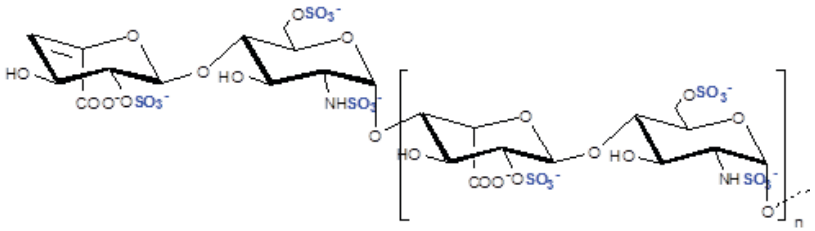


Fig. 39: General formula of heparin oligosaccharide:
ΔUA, 2S-GlcNS, 6S-(IdoA, 2S-GlcNS, 6S)n-IdoA, 2S-GlcNS, 6S

Uronic acid (ΔUA) is the non-reducing end of oligosaccharides. n is the number of disaccharide units, n=0 is the dp4 tetrasaccharide, n=1 is the dp6 hexasaccharide, etc.

Table 9. Selected examples of specific protein-binding sequences in heparin/heparan sulfates

GAG-sequence	Interacting protein	References
GlcNAc/NS(6S)-GlcA-GlcNS(3,6S)-IdoA(2S)-GlcNS(6S)	Antithrombin III	Oscarsson LG 1989 JBC
UA, 2S-GlcNS,6S-(IdoA, 2S-GlcNS, 6S)$_2$-GlcA-GlcNS,6S-	ApoE	Bazin HG 2002 Biochemistry
UA-GlcNS-IdoA(2S)-GlcNAc-UA(2S)-GlcNS-IdoA(2S)-GlcNH2(3,6S)	Herpes Simplex Virus-1 gD	Liu J 2002 JBC
IdoA-GlcNAc(6S)-GlcA-GlcNS(3,6-S)-IdoA(2S)-	Heparanase	Gong F 2003 JBC
-IdoA2S-GlcNS6S-	HSulf1, HSulf2	Morimoto-Tomita 2002 JBC
GlcNS-IdoA(2S)-GlcNS(6S)-IdoA(2S)GlcNS	VEGF 165	Robinson CJ 2006 JBC
GlcA-GlcNS-[IdoA(2S)-GlcNS]$_5$-IdoA GlcNAc	FGF2	Turnbull JE 1992 JBC
-IdoA(2S)-GlcNS(6S)-IdoA(2S)-	FGF1	Kreuger J 2001 JBC
UA-GlcNS-[UA(2S)-GlcNS(6S)]$_3$	FGF4, FGF7	Ashikari S 2004 JBC
UA-GlcNS-[UA-GlcNS(6S)]$_3$	FGF10	Ashikari S 2004 JBC
UA-GlcNS-[UA(2S)-GlcNS]$_3$, UA-GlcNS-[UA-GlcNS(6S)]$_3$	FGF18	Ashikari S 2004 JBC
-GlcA-GlcNAc/S-[IdoA(2S)-GlcNS(6S)]$_3$-[GlcA-GlcNAc/S]6-7-[IdoA(2S)-GlcNS(6S)]$_3$-GlcA-GlcNAc/S	IL18	Spillmann D 1998 JBC
IdoA(2S)-GlcNH2(3S 6S) within dp8	CyPB	Vanpouille C 2007 JBC

OBJECTIVES

OBJECTIVES

Viral entry process relies on a fine interplay between the virion and a host cell. Infection is initiated by interaction of the viral particle with specific proteins on the cell surface. These proteins belong to two general categories depending on the functional consequences of the interaction: attachment factors and receptors. Attachment factors serve to bind and concentrate particles at the cell surface. Usually, these interactions have a rather low specificity. On the other hand, receptors actively promote virus entry by inducing conformational changes of the viral particle and/or by activating signaling pathways or internalization of the virion. Currently, HCV entry is viewed as a complex multistep process as at least four major specific cellular entry factors have been shown to be essential in the early steps of the HCV life cycle. These molecules are SRB1, the tetraspanin CD81 and tight junction proteins, CLDN1 and OCLN. The involvement of these four molecules in HCV entry is now well documented and their role at some particular steps of HCV entry begins to be partly understood. However, much less is known about the early steps of HCV entry. In particular, the initial attachment of HCV particle to host cell-surface remains poorly understood.

As a result of HCV virion interaction with lipoproteins, the LDL receptor (LDLr) has first been proposed as a potential attachment factor for HCV, allowing for the initial binding of HCV particles to host cells (Agnello *et al*., 1999). However, HCV-LDLr interaction seems to involve a non-productive entry pathway that can potentially lead to viral particle degradation as suggested (Albecka *et al*., 2012). Besides the LDLr, HSPGs have also been proposed as HCV attachment factors (Germi *et al*., 2002; Koutsoudakis *et al*., 2006; Morikawa *et al*., 2007; Molina *et al*., 2007). Indeed, HSPGs are often used by viruses as attachment factors, and the matrix of the space of Disse and the surface of hepatocytes are rich in HSPGs. However, studies to characterize the potential involvement of HS in virus entry have been initiated before the development of a cell culture system for this virus, and they were based on the assumption that, as observed for other viruses, envelope proteins present at the surface of the virion should contain HS-binding site (s) if the virus needs HS to initiate its life cycle. Based on these studies, it was reported that HCV envelope proteins interact with HSPGs and that HVR1 plays a role in this interaction (Barth *et al*., 2003; Barth *et al*., 2006). However, other components of the viral particle can also interact with HS molecules. Indeed, apoE, which is found at

the surface of HCV virion, is known to contain HS-binding motifs, and it has been proposed that this apolipoprotein could be the viral component responsible for virion binding to HSPGs (Jiang *et al*., 2012; Jiang *et al*., 2013; Lefévre *et al*., 2014). In contrast, another study in the context of the HCVcc system rather suggested the implication of HVR1 in the initial binding to HSPGs (Koutsoudakis *et al*., 2012). It remained therefore important to determine the relative contribution of HCV envelope proteins and apoE in the early events of HCV entry, specifically in the HSPG-binding step. Furthermore, it is important to note that structural determinants of HS required for HCV infection have also been studied with the help of surrogate models and not in the context of the infectious cell culture system.

The objective of this thesis was therefore to characterize the interaction of HCV particle with cell-surface HSPG. Specifically, our first goal was to clarify the relative contribution of viral components: HCV envelope protein E2 (primarily the HVR1 of E2) and virion-associated apoE in the interaction with HS, in the context of the HCVcc system. The second aim of this work was also to characterize the structural determinants of HS required for HCV infection.

RESULTS

RESULTS

Characterization of hepatitis C virus interaction with heparan sulfate proteoglycans

Yan Xu[1], Pierre Martinez[2], Karin Séron[1], Guangxiang Luo[3], Fabrice Allain[2], Jean Dubuisson[1#], Sandrine Belouzard[1#*]

[1] Center for Infection & Immunity of Lille (CIIL), Inserm U1019, CNRS UMR 8204, Institut Pasteur de Lille, University of Lille Nord de France, F-59000 Lille, France.

[2] Unité de Glycobiologie Structurale et Fonctionnelle, CNRS UMR 8576, Université Lille Nord de France - Université des Sciences et Technologies, 59655 Villeneuve d'Ascq, France.

[3] Department of Microbiology, University of Kentucky College of Medicine, Lexington, Kentucky, United States of America

Running title : HCV and HS

**Corresponding author: Dr Sandrine Belouzard, Molecular & Cellular Virology of Hepatitis C, CIIL, Inserm (U1019) & CNRS (UMR8204), Institut Pasteur de Lille, Bâtiment IBL, 1 rue Calmette, BP447, 59021 Lille cedex, France. Fax: (+33) 3 20 87 12 01; E-mail:* Sandrine.belouzard@ibl.cnrs.fr

# JD and SB contributed equally to this paper

❖ Manuscript in preparation

ABSTRACT

Hepatitis C virus (HCV) entry involves binding to cell surface heparan sulfate (HS) structures. However, due to the lipoprotein-like structure of HCV, the exact contribution of virion components to this interaction remains controversial. Here, we investigated the relative contribution of HCV envelope proteins and apolipoprotein E in the HS-binding step. Deletion of hypervariable region 1, a region previously proposed to be involved in HS-binding, did not alter HCV virion binding to HS, indicating that this region is not involved in this interaction. Neutralizing monoclonal antibodies recognizing different regions of HCV envelope glycoproteins were also used in a pull-down assay with beads coated with heparin, a close HS structural homologue. Although isolated HCV envelope glycoproteins could interact with heparin, none of these antibodies was able to interfere with virion-heparin interaction, strongly suggesting that, at the virion surface HCV envelope glycoproteins are not accessible for HS binding. In contrast, results from kinetic studies, heparin pull-down and inhibition experiments with anti-apolipoprotein E antibodies indicate that this apolipoprotein plays a major role in HCV-HS interaction. Finally, characterization of HS structural determinants required for HCV infection by silencing enzymes involved in the HS biosynthesis pathway and by competition with modified heparin indicated that *N*- and 6-*O*-sulfation but not 2-*O*-sulfation are required for HCV infection, and that the minimum HS oligosaccharide length required for HCV infection is a decasaccharide. Together, these data indicate that HCV hijacks apolipoprotein E to initiate its interaction with specific HS structures.

INTRODUCTION

HCV belongs to the genus Hepacivirus in the *Flaviviridae* family (Stuart C. Ray, 2013). It is a small enveloped virus with a positive single stranded RNA genome of 9.6 Kb. The genome is translated as a polyprotein of ~ 3000 amino acids, which is processed during translation by cellular and viral proteases to generate the structural (capsid, E1 and E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Lindenbach et al., 2005). The structural proteins are components of the viral particle. By being present at the surface of the viral particle, HCV envelope glycoproteins E1 and E2 play a major role in HCV entry (Bartosch et al., 2003; Hsu et al., 2003). These glycoproteins are type I transmembrane proteins which form a noncovalent heterodimer within infected cells, whereas they assemble as large covalent complexes stabilized by disulfide bonds on the viral particle (Vieyres, Dubuisson, and Pietschmann, 2014). Within E1E2 complex, E2 is currently the best-characterized subunit (Vieyres, Dubuisson, and Pietschmann, 2014). Indeed, it is the major target of neutralizing antibodies (Ball, Tarr, and McKeating, 2014), and it is also the receptor-binding protein, which has been shown to interact with tetraspanin CD81 (Pileri et al., 1998) and scavenger receptor BI (SRB1)(Scarselli et al., 2002).

A striking and unique feature of HCV biology is the unusually low buoyant density of the virion, which results from its physical association with lipoproteins, forming a hybrid particle called lipoviroparticle (LVP) (Andre et al., 2002). Due to virion association with lipoproteins, apolipoproteins such as apoE, apoB, apoA1, apoC1, apoC2 and apoC3 can also be found in association with HCV particles (Bartenschlager et al., 2011; Catanese et al., 2013). Furthermore, a characterization of cell culture-produced particles indicates that their lipid composition resembles the one of very-low density lipoproteins (VLDL) and low-density lipoproteins (LDL) (Merz et al., 2011). Among HCV-associated apolipoproteins, there is a consensus about the involvement of apoE in HCV morphogenesis (Coller et al., 2012; Hueging et al., 2014; Jiang and Luo, 2009).

HCV entry is a complex process involving many cellular partners and viral components. Indeed the initial attachment of the virus is followed by a series of sequential interactions with numerous host factors, internalization of the viral particle by clathrin-mediated endocytosis and fusion of the viral envelope with endosomal membranes (Zeisel, Felmlee, and Baumert, 2013). It is now well established that heparan sulfate proteoglycans (HSPG) serve as primary docking sites for many viruses. In the case of HCV, syndecan 1 has been reported to be a major

attachment factor (Shi, Jiang, and Luo, 2013). This initial attachment of the virus to HSPG and potentially the low-density lipoprotein receptor, is followed by the sequential interactions with at least four specific cellular entry factors: the scavenger receptor SRB1, the tetraspanin CD81 and two tight junction proteins: claudin-1 (CLDN1) and occludin (OCLN). In the recent years, many cellular factors participating or regulating different steps of the entry process were identified, these factors include the epithelial growth factor receptor (EGFR) (Lupberger et al., 2011), the Niemann-Pick type C1 like 1 (NPC1L1) cholesterol uptake receptor (Sainz et al., 2012) and the transferrin receptor 1 (TSFR1) (Martin and Uprichard, 2013).

HSPG are abundant in the matrix of the space of Disse and at the surface of hepatocytes. They are composed of a core protein and heparan sulfate (HS) chains, which are linear polysaccharides consisting of a repeated disaccharide unit of an uronic acid and a derivative of glucosamine with various sulfation patterns (Esko and Selleck, 2002). It has first been reported that viral particles isolated from patients interact with glycosaminoglycans (GAGs)(Germi et al., 2002). Following this observation, it has been shown that recombinant HCV envelope glycoprotein E2 as well as virion-associated glycoprotein complexes interact with HSPG, suggesting a direct contact between the viral components of the virion and HSPG (Barth et al., 2003; Vieyres et al., 2010). Furthermore, E2 hypervariable region 1 (HVR1) has been proposed to contribute to this interaction (Barth et al., 2003; Barth et al., 2006; Basu et al., 2007; Koutsoudakis et al., 2012). On the other hand, apoE, which is found on the surface of LVPs, is also able to interact with HSPG, and it has recently been reported that this apolipoprotein could be responsible for the binding of HCV virions to HSPG (Jiang et al., 2012; Jiang et al., 2013). Here, we investigated the relative contribution of HCV envelope proteins and apoE in the early event of HCV entry, specifically in the HSPG-binding step, and we characterized the structural determinants of HS required for HCV infection. Our results support the hypothesis that apoE associated with the virus is likely responsible for HCV-HS interaction. We also found that *N*- and 6-*O*-sulfation but not 2-*O*-sulfation of HS are required for HCV infection. Finally, we show that the minimal HS length unit required for HCV infection is a decasaccharide.

MATERIALS AND METHODS

Cell culture. The Huh-7 hepatoma cell line (Nakabayashi et al., 1982) was cultured in Dulbecco's Modified essential medium (DMEM; Life Technologies) supplemented with 0.1 mM

non-essential amino acids (NEAA, Life Technologies) and 10% fetal bovine serum (Life Technologies).

Antibodies and reagents. Mouse anti-HCV monoclonal antibodies (Mabs) A4 (anti-E1) and A11 (anti-E2) (Dubuisson et al., 1994), rat anti-HCV Mabs 9/27 and 3/11 (anti-E2, kindly provided by JA McKeating, University of Birmingham, United Kingdom)(Hsu et al., 2003) and human anti-HCV AR3A and AR5A (anti-E2 and anti-E1E2 respectively, kindly provided by M Law, The Scripps Research Institute, La Jolla, USA) (Giang et al., 2012) were used in this work. ApoE-specific Mab 23 has been previously described (Chang et al., 2007). Control human Mab RO4 was kindly provided by S Foung (Stanford University). Polyclonal anti-apoE antibody was from Millipore. Soluble heparin extracted from bovine lungs was purchased from (Sigma-Aldrich) and desulfated heparins from AMS Biotechnology (UK). ApoE-derived peptide (LRKLRKRLLLRKLRKRLL) was synthesized by ProteoGenix.

Preparation of heparin oligosaccharides. Heparin-derived oligosaccharides were obtained as described previously (Vanpouille et al., 2004). Briefly, 100 mg of heparin were incubated with 50 units of heparinase I (Iduron) at 30°C for 30 h. After desalting on a Sephadex G-10 column (Pharmacia Amersham Biotech), the digestion mixture was fractionated by filtration on Bio-Gel P-6 (Bio-Rad Laboratories). Pooled fractions corresponding to increasing degree of polymerization (dp) oligosaccharides were eluted by 0.2 M NH_4Cl, pH 3.5, desalted and freeze-dried.

Plasmid constructions. The virus used in this study is based on the JFH-1 strain (genotype 2a; GenBank accession number AB237837) (Wakita et al., 2005), kindly provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan). In addition, an intergenotypic H77/JFH1 chimera was also used in some experiments (Maurin et al., 2011). Deletions were introduced in a modified version of the plasmid carrying the full length JFH-1 genome (pJFH1-CS-A4) engineered to reconstitute the A4 epitope in E1 (Goueslain et al., 2010) and titer-enhancing mutations(Delgrange et al., 2007). The deletion of the hypervariable region 1 was introduced in the plasmid carrying the full-length genome by PCR. Two PCR products were generated by using the following primers Sense1 (5’CGTACGTGATGCGCGTCCCCG3’) and Anti-Sense1 (5’CTGCCGTTGGTGTTAATGAGCTGAATCGCGTCCACCCCAGCGGCCAG3’), Sense2 (5’CTGGCCGCTGGGGTGGACGCGATTCAGCTCATTAACACCAACGGCAG3’) and Anti-Sense2 (5’ GGTACCCACTCCTGAATCATGG 3'). The two fragments were assembled by a

second PCR amplification by using the primers Sense 1 and Anti-sense 2. The PCR product was ligated in the pJFH1-CS-A4 after digestion with *Bsi*WI and *Kpn*I (New England Biolabs). The nucleotide sequence was verified by sequencing.

Virus production and purification. Plasmids encoding the wild type or HVR1 deleted viruses were digested with *Xba*I and treated with the Mung bean nuclease (New England Biolabs). *In vitro* transcriptions were performed using the Megascript kit (Ambion) according to the manufacturer's protocol. Viruses were rescued by electroporation of Huh7 cells with 10 µg of *in vitro* transcribed RNA as described in (Delgrange et al., 2007). Viral supernatants were collected and viruses were amplified to obtain viral stocks. For virus purification, highly infectious viral supernatants were collected and cleared by centrifugation. Viruses were concentrated by overnight precipitation with 8 % polyethylene glycol (Fluka Chemie AG) and centrifugation at 13,000xg for 20 minutes. Pellets were resuspended in 1ml of cold PBS and layered on the top of a 10-50% continuous iodixanol gradient (Optiprep, Proteogenix). Gradients were ultracentrifuged for 16h at 160,000xg at 4°C in a SW41 rotor. Twelve fractions of 1 ml were collected and the two most infectious fractions were used for experiments involving purified virus.

Indirect immunofluorescence. HCV infected-cells grown on glass coverslips were fixed in 100% -20°C cold methanol. After two washes with PBS, cells were blocked with 10% goat serum in PBS. Infected cells were detected by using anti-E1 Mab A4 and visualized with cyanine 3-conjugated goat anti-mouse IgG secondary antibodies (Jackson Immunoresearch). Cell nuclei were stained with 4',6'-diamidino-2-phenylindoledium (DAPI). Coverslips were observed with a Zeiss Axiophot microscope equipped with 10× magnification objective (Carl Zeiss AG, Oberkochen, Germany). Fluorescent signals were collected with a Coolsnap ES camera (Photometrix, Kew, Australia). For quantification, images of randomly picked areas from each coverslip were recorded.

Heparin pull-down assay of HCV envelope glycoproteins. Infected cells or purified viruses were lysed with PBS-1% Triton X100 in the presence of protease inhibitors (Complete, Roche Diagnostics). Lysates were incubated overnight at 4°C with heparin-sepharose (CL-6B). Then, the beads were washed 5 times with 1% Triton-X100 in PBS. After the last wash, the beads were resuspended in Laemmli loading buffer and incubated 10 minutes at 70°C. Protein samples were separated by SDS-PAGE and then were transferred onto nitrocellulose membranes (Hybond-ECL,Amersham). Proteins were probed with specific antibodies and corresponding peroxidase

conjugated anti-species antibodies (anti-mouse and anti-rat, Jackson immunoresearch). Peroxidase activity was detected by chemoluminescent reaction (Pierce ECL substrate, Thermo Scientific)

Heparin pull-down assay of viral particles. Purified virus was incubated in the presence or absence of different competitors (25 µg/ml heparin, 5 µg/ml Mab AR3A, 10 µg/ml Mab AR5A, 10 µg/ml Mab RO4, 20 µl Mabs 9/27, 1/200 anti-apoE polyclonal antibody or 20µg/ml apoE-derived peptide) in PBS for 1h at 37°C. Then, the mixture was cooled down and incubated for 2h at 4°C with heparin-sepharose beads. The beads were washed three times with cold PBS and RNAs were extracted by using the QIAamp Viral RNA kit (Qiagen). HCV genomes were quantified by real-time quantitative PCR as described previously (Castelain et al., 2004).

Virus attachment and infection assay. Purified virus was pre-incubated with or without heparin or antibodies in DMEM-Hepes (DMEM without bicarbonate containing 25 mM HEPES buffer) for 1h at 37°C. Virus preparations were then cooled down and incubated with target cells for 2h at 4°C. Cells were rinsed with PBS. For binding measurement, total RNA from cell lysates was extracted by using the Nucleospin RNA kit (Macherey-Nagel) as recommended by the manufacturer. For infection assay, infected cells were further incubated for 30h at 37°C, fixed and processed for immunofluorescence.

Real-time PCR of HS sulfotransferases. Total RNA was isolated from 4.10^6 Huh7 cells by using the Nucleospin RNA kit (Macherey-Nagel) as recommended by the manufacturer. Reverse transcription was performed from 1 µg of total RNA by using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Synthetic primers for *N*-deacetylases-*N*-sulfotransferases 1-4 (NDST1, NDST2, NDST3, NDST4) and 3-*O*-sulfotransferases 1, 2, 4, 5, 6 (3-OST1, 3-OST2, 3-OST4, 3-OST5, 3-OST6) were described in (Deligny et al., 2010) and for 2-*O*-sulfotransferase (2-OST), 3-OST3A, 3-OST3B and 6-*O*-sulfotransferases (6-OST1, 6-OST2, 6-OST3) in (Gotte et al., 2007). Real-time PCR amplifications were performed using an Mx3000P Multiplex Quantitative PCR system (Stratagene). The transcript of HPRT was used as a control to normalize the expression of genes of interest. Each PCR reaction consisted of 25 µl containing 2 µl of diluted cDNA sample (1:5), 12.5 µl of Maxima SYBR Green qPCR Master Mix (two times) (Thermo Scientific), 1 µl of forward primer (7.5 µM for 2-OST; 22.5 µM for 3-OST1 and 3-OST2; 15 µM for all the others), 1 µl of reverse primer (7.5 µM for 2-OST; 22.5 µM for NDST4 and 3-OST6; 15 µM for all the others) (all primers from Eurogentec), and 8.5 µl of water. It also included a non-template negative control to check for primer dimers. The

conditions of PCR were as follows: 1 cycle of denaturation at 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at specific temperature of annealing (67 °C for NDST4, 3-OST1 and 3-OST4; 68°C for 3-OST6; 60°C for all the others) and 30 s at 72 °C. The fluorescence data were measured at the end of each cycle. A melting curve (55–95 °C at 1 °C interval) was constructed for each primer pair to check for the presence of one gene-specific peak. The amplification efficiency of each primer pair was performed on serial dilutions of cDNA. Triplicate PCRs were prepared for each sample. The point at which the PCR product is first detected above a fixed threshold, termed cycle threshold (Ct), was determined for each sample, and the average Ct of triplicate samples was used for further analysis. The relative quantification of transcripts was calculated as described previously (Pfaffl, 2001).

RNA interference. Huh-7 cells were transfected with siRNA pools (Dharmacon) targeting CD81 (CACGUCGCCUUCAACUGUA), NDST1 (CCUCCGACUUCUACUUUGA), 2-OST (On target plus smart pool) or 6-OST1 (CCAGGAAGUUCUACUACA) and 6-OST2 (On target plus smart pool) using RNAiMax (Invitrogen) and according to the manufacturer's instructions. Briefly, 3 µl RNAiMAx lipofectamine in 500 µl PBS were mixed with 50 pmol siRNA per well in 6-well plates. After 30 minutes of incubation, 2 ml of complete medium containing $2x10^5$ cells were added to the siRNA/lipofectamine mix for each well. The knock down effects were determined at 96h after transfection by Western blot or quantitative RT-PCR analysis at the time of virus inoculation. For infection assay, the cells were incubated for 1h with virus. The effects of silencing on virus infection were determined 30 h after inoculation by immunolabeling of the infected cells.

Graphs and statistics. Prism v5.0c (GraphPad Software Inc., La Jolla, CA) software was used to prepare graphs and to determine statistical significance of differences between data sets.

RESULTS

HVR1 is not the main determinant of HCV-interaction with HS. Due to a high level of *N*-linked glycosylation, the surface of HCV envelope glycoproteins available to interact with HSPG is reduced (Helle et al., 2010). However, in agreement with its high level of sequence variability, HVR1 is believed to be accessible at the surface of the viral particle, which fits with the hypothesis that this region might play a role in HCV interaction with HSPG (Barth et al., 2003; Barth et al., 2006; Basu et al., 2007; Helle et al., 2010; Koutsoudakis et al., 2012). We therefore reassessed the role of HVR1 in HCV interaction with HSPG. To this end, we first

produced a HVR1-deleted virus in the context of the JFH1 strain (JFH1-ΔHVR1). This virus was amplified and purified to obtain infectious viral stocks with titers similar to the wild-type viruses. The viral stock of JFH1-ΔHVR1 was sequenced to be sure that no adaptive mutation was present in the E1E2 region. It is worth noting that both purified JFH1 and JFH1-ΔHVR1 had a similar specific infectivity (data not shown). However, as previously reported (Bankwitz et al., 2010; Prentoe et al., 2011), a slight difference in buoyant densities was observed (data not shown).

To investigate the binding properties of the JFH1-ΔHVR1 virus, we first determined its capacity to interact with hepatoma cells. Huh-7 cells were therefore incubated at 4°C with similar amounts of purified JFH1 and JFH1-ΔHVR1 viruses, and bound viral particles were determined by measuring HCV RNA associated with the cells after removing unbound virions. As shown in Figure 1A, JFH1-ΔHVR1 virus had a binding capacity similar to the wild-type virus, indicating that HVR1 is not essential for initial binding of HCV particles to Huh-7 cells. Next we determined the capacity of heparin, a close structural homologue of highly sulfated HS, to inhibit viral attachment to Huh-7 cells in a competition assay. As shown in Figure 1B, JFH1-ΔHVR1 virus was inhibited by heparin in a dose-dependent manner, indicating that this virus still relies on HS binding to initiate cell infection. It is worth noting that JFH1-ΔHVR1 is even more sensitive than the wild-type virus to heparin competition. Indeed, a residual infectivity of 40% was observed for JFH1 at the highest heparin concentrations, whereas it decreased to approximately 20% for JFH1-ΔHVR1 virus. These data suggest that the HVR1 region of E2 is not involved in virus binding to HS.

To determine whether E2-ΔHVR1 interacts with HS, we performed heparin pull-down experiments with lysates of cells infected JFH1 or JFH1-ΔHVR1 viruses. As shown in Figure 2A, HCV envelope glycoproteins from these two viruses were equally pulled down, indicating that HVR1 is not necessary for cell-associated E1E2 binding to heparin. However, since dramatic reorganization of HCV envelope glycoproteins occurs during virion budding and/or egress (Vieyres et al., 2010), we also performed heparin pull-down experiments with lysates of purified JFH1 or JFH1-ΔHVR1 viruses. As shown in Figure 2B, HCV envelope glycoproteins from these two virions were equally pulled down, indicating that HVR1 is not necessary for virion-associated E1E2 binding to heparin. Taken together, these data indicate that the HVR1 region of E2 is not the main determinant for cell surface attachment and binding to HSPG, however other regions of the viral glycoproteins can contribute to HS binding in the context of isolated proteins.

ApoE is involved in HCV binding to the cell surface. Due to the presence of envelope glycoproteins as well as apolipoproteins at the surface of HCV viral particle, contradictory results have been obtained concerning the virion determinant involved in the initial step of binding to host cells (Barth et al., 2003; Barth et al., 2006; Basu et al., 2007; Jiang et al., 2012; Jiang et al., 2013; Koutsoudakis et al., 2012). Indeed, both HCV envelope glycoprotein E2 and apoE have been proposed to be the virion component involved in binding to target cells. Therefore, to further characterize our JFH1-ΔHVR1 mutant, we determined its sensitivity to neutralization by anti-apoE antibodies. As shown in Figure 3, both JFH1 and JFH1-ΔHVR1 viruses were inhibited by anti-apoE antibody. However, JFH1-ΔHVR1 was more sensitive than the wild-type virus to neutralization by anti-apoE antibody. Indeed, a residual infectivity of 60% was observed for JFH1, whereas it went down to approximately 40% for JFH1-ΔHVR1 virus.

Due to the involvement of several cellular entry factors in HCV entry, kinetic studies of inhibition by specific antibodies have been used to determine the sequential implication of these different entry factors (Koutsoudakis et al., 2006; Zeisel, Cosset, and Baumert, 2008). This can easily be done by incubating the virus with host cells at 4°C and then shifting the temperature to 37°C, with the specific antibodies or inhibitors added at different time points (Figure 4A). We therefore performed this type of experiment to determine whether apoE is involved in an early step of HCV entry. In parallel, we used heparin, which is known to inhibit the initial step of virion binding. As shown in Figure 4A, the maximum inhibitory effect of anti-apoE antibody was observed when it was added together with the virus at 4°C. In contrast, no inhibition was observed if the virus was incubated with host cells at 4°C prior to treatment with the anti-apoE antibody. These results are very similar to the inhibitory effects observed with heparin, suggesting that apoE antibody inhibits HCV attachment at the cell surface. To confirm that the anti-apoE antibody inhibits virion attachment to host cells, Huh-7 cells were incubated at 4°C with purified JFH1 virus pre-incubated or not with a specific antibody or heparin and bound viral particles were determined by quantifying HCV RNA associated with the cells after removing unbound virions. As shown in Figure 4B, the anti-apoE antibody inhibited virion binding to host cells. Together, these data indicate that apoE present at the surface of the viral particle is involved in virion attachment to host cells, which could likely be due to its binding to HSPG.

ApoE is responsible for HCV interaction with HS. Although our data do not support a role for HVR1 in HCV particle interaction with HS, we cannot definitively exclude that other regions in HCV envelope glycoproteins exposed on the virion are involved in binding to HSPG. Furthermore, although our data indicate that apoE is involved in HCV virion binding to host

cells, we still needed to confirm that virion-associated apoE directly binds to HS. Therefore, to clarify the relative contribution of apoE and envelope glycoproteins in HCV interaction with HS, we performed pull-down experiments with heparin-coated beads in the presence of competitors that target apoE-HS interaction or in the presence of different neutralizing anti-HCV antibodies. Because the antibody 9/27 is directed against the E2 envelope protein of the genotype 1a, we used a H77/JFH1 chimera for this experiment. As shown in Figure 5, none of the anti-HCV neutralizing antibodies inhibited HCV binding to heparin. Interestingly, the epitope that is recognized by the neutralizing antibody 9/27 is located in HVR1 (Hsu et al., 2003), however this antibody was unable to inhibit the binding of the virus to heparin beads confirming that the HVR1 is not crucial for virus binding to HS. To exclude that other regions of envelope glycoproteins can mediate the interaction with HS, we also used Mab AR5A, a conformational neutralizing antibody that recognizes a discontinuous epitope on E1E2 heterodimer, and Mab AR3A, another conformational neutralizing antibody that disrupts E2 binding to CD81 (Giang et al., 2012). However neither AR5A nor AR3A was able to compete with HCV virion binding to heparin. In contrast, pre-incubation of the virions with anti-apoE antibody strongly inhibited HCV binding to heparin beads. Furthermore, incubation with an HS binding peptide derived from apoE also markedly inhibited interaction of HCV virions with heparin beads. Together, these results are in favor of a major role of apoE in HCV binding to HS.

HS structural determinants involved in HCV binding. To address the HS structural determinants important for interaction with HCV, we first analyzed the minimal HS length unit required to inhibit HCV infection by using heparin-derived oligosaccharides of a defined length. As shown in Figure 6, we observed a 50% decrease of infection in the presence of heparin, and pre-incubation of the virus with oligosaccharides of dp10 or dp12 also inhibited HCV infection at levels similar to heparin. These data suggest that a minimum of 10 saccharides is necessary for HCV virion interaction with HS.

To further characterize HS determinants involved in HCV binding, we identified HS sulfotransferases expressed in Huh-7 cells. HS biosynthesis is divided in 3 main steps: chain initiation, polymerization and modification. The initiation step is characterized by the linkage of a tetrasaccharide to the proteoglycan core protein. Then, HS backbone is formed by the assembly of alternating glucuronate (GlcUA) and *N*-acetylglucosamine (GlcNAc) residues. As the chain assembles, it undergoes a series of modifications catalyzed by a C_5 epimerase and multiple sulfotransferases. The first modification of the chain to occur is the removal of *N*-acetyl group from subsets of GlcNAc and the addition of *N*-sulfo group. This reaction is orchestrated by

members of the NDST family. Further modifications of HS include epimerization of some glucuronate to iduronate residues, addition of sulfate groups at C_2 of uronic acid by 2-OST, and at C_6 and/or at C_3 of GlcN residues by 6-OST and 3-OST, respectively. Although 2-OST is represented by a unique isoform, 6-*O*-sulfation and 3-*O*-sulfation of HS can be catalyzed by three 6-OSTs and seven 3-OSTs. We therefore analyzed the expression profile of isoenzymes that are involved in HS sulfation. As shown in Figure 7, we detected high levels of mRNA encoding NDST1 in Huh-7 cells. The expression of NDST2 was very low, while NDST3 and NDST4 transcripts were not detected. We also found a high level of 2-OST transcripts and a modest expression of 6-OST1 and 6-OST2, both isoenzymes being involved in the same 6-*O*-sulfation reaction. Even though seven enzymes are involved in the 3-*O*-sulfation reaction, we only detected low level of 3-OST3A, 3-OST3B and 3-OST5 in Huh7 cells.

To determine if particular pattern of sulfation are important for HCV-HS interactions, we performed infection in the presence of chemically modified heparins. As shown in Figure 8, the 2-*O*-desulfated heparin was able to inhibit HCV infection by 50% whereas unmodified heparin inhibits 80% of HCV infection. In contrast, *N*- or 6-*O*-desulfated heparins used at the same concentrations were unable to inhibit HCV infection. These results suggest that *N*- and 6-*O*-sulfo groups are important for HCV infection. To confirm these results, we used a silencing approach. We based our silencing strategy on the expression profile of the enzymes expressed in Huh-7 cells. Consequently, small RNAs interfering with the expression of NDST1, 2-OST and a combination of siRNAs targeting the two isoforms of 6-OST (6-OST1 and 6-OST2) were used. Furthermore, CD81 silencing was used as a positive control. The down-regulation of the mRNA levels of the different enzymes was analyzed at 72 h post-transfection. The mRNA levels of NDST1, 2-OST and 6-OST2 were efficiently decreased by their specific siRNAs (81 ± 7 %, 85 ± 7 % and 74 ± 15 %, respectively). Although we tested different siRNAs for 6-OST1, we could not achieve more than 60% of silencing for this enzyme. It is worth noting that in cells silenced for the expression of NDST1, a 1.5- to 2-fold upregulation of 6-OST1 was observed (data not shown). Then, we analyzed the consequence of the silencing of the different enzymes on HCV infection. As shown in Figure 9, infection was severely inhibited in CD81 silenced cells. We observed a 60% decrease of HCV infection in cells silenced for NDST1, indicating that *N*-sulfation is necessary for HCV interaction with GAGs. HCV infection was unaffected by the silencing of 2-OST, whereas the silencing of both isoforms of 6-OST reduced HCV infection. We didn't investigate the role of 3-*O*-sulfation in HCV entry because its requirement is very unlikely. Indeed, the most expressed 3-*O*-sulfatase in Huh7 cells is 3-OST3B, an enzyme that

relies on prior 2-*O*-sulfation of the HS chain for its activity and our results indicate that the 2-OST silencing has no effect on HCV infection. Together, our data are in agreement with the results obtained with the desulfated heparins and support the idea that *N*- and 6-*O*-sulfation but not 2-*O*-sulfation are required for HCV infection.

DISCUSSION

As many viruses, HCV interacts with HS, a complex group of cell surface associated anionic polysaccharides. However, the exact contribution of HCV virion components to this interaction remains controversial. This is due to the unique nature of HCV particle, which is associated with lipoproteins. Indeed, both apoE and HCV envelope glycoproteins have been proposed to play a role in HS binding (Barth et al., 2003; Barth et al., 2006; Basu et al., 2007; Jiang et al., 2012; Jiang et al., 2013; Koutsoudakis et al., 2012). However, after reinvestigating this question, our data support a major role played by apoE in HS binding, whereas the viral envelope glycoproteins present at the surface of the virion do not seem to be involved. We also characterized the structural determinants of HS that contribute to this interaction, and we found that *N*- and 6-*O*-sulfation but not 2-*O*-sulfation is required for HCV infection. Finally, we show that the minimum HS length unit required for HCV infection is a decasaccharide.

HVR1 sequence of E2 glycoprotein is not involved in the binding of HCV envelope glycoproteins to HS. Before the development of a cell culture system for HCV, the potential role of HS in HCV entry was first proposed based on experiments performed with viral particles isolated from patients (Germi et al., 2002). However, in the absence of a culture system for HCV, recombinant viral envelope glycoproteins or surrogate models of viral particles have also been used to study this initial step of the HCV life cycle. Recombinant HCV envelope glycoprotein E2 was initially reported to interact with HS (Barth et al., 2003), and the capacity of HCV envelope glycoproteins to interact with HS has been confirmed with proteins isolated from purified virus (Vieyres et al., 2010). Furthermore, based on studies with recombinant proteins or pseudoparticles containing HCV envelope glycoproteins, HVR1 region of E2 has been proposed to contribute to this interaction (Barth et al., 2003; Barth et al., 2006; Basu et al., 2007). Here, we used a functional and a biochemical approach to reinvestigate the potential contribution of HVR1 to HCV glycoproteins interaction with HS. However, neither the functional experiments nor the biochemical approach confirmed a role for HVR1 in this interaction. For the biochemical data, the discrepancy could be due to the fact that the first observation was made in the context of a truncated recombinant E2 protein expressed alone (Barth et al., 2003), whereas in our case,

we used E1E2 complexes produced by a replicative virus. Concerning the functional studies, previous observations were first made with the help of pseudoparticles (Barth et al., 2006; Basu et al., 2007). However, these model systems are not the most appropriate tools to study such interactions since pseudoparticles devoid of any viral envelope protein can directly bind to target cells (Pizzato et al., 1999), and such envelope-independent binding can be inhibited by heparin (Walker et al., 2002). Other experiments performed with the HCVcc system also suggested a role for HVR1 in HCV binding to HS (Koutsoudakis et al., 2012). However, in this case only functional studies were performed without biochemical confirmation, and the phenotype observed could be due to indirect effects of the mutations, since HVR1 is involved in interaction with SRB1 (Scarselli et al., 2002).

HCV envelope glycoproteins do not seem to play a role in the initial interaction of HCV particles with HS. The protein surface of HCV envelope glycoproteins is poorly exposed at the surface of viral particles this is due in part to the association with lipoproteins (Merz et al., 2011) and to the presence of a large number of *N*-linked glycans (Helle et al., 2010; Kong et al., 2013). These features dramatically reduce the possibilities for these glycoproteins to interact with HS when associated with the viral particle. They also explain the limited number of neutralizing epitopes identified on HCV glycoproteins. However, such neutralizing antibodies are interesting tools to probe the potential interaction of HCV envelope glycoproteins for their interaction with HS in the context of the virion. We therefore used several neutralizing antibodies targeting different regions of HCV envelope glycoproteins to determine their capacity to compete with HCV binding to heparin. As shown with Mab AR3A, the CD81-binding region is not involved in HCV-HS interaction, which is in agreement with a similar observation made with another Mab targeting this region (Jiang et al., 2012). HVR1 is the most virion-exposed region of HCV envelope glycoproteins, and in agreement with the data obtained with our HVR1-deletion mutant, Mab 9/27, recognizing an epitope in this region (Hsu et al., 2003), does not inhibit HCV-HS interaction. Finally, we also used a neutralizing antibody recognizing an epitope shared by E1 and E2 and located outside of the CD81-binding region (Giang et al., 2012), and again no competition was observed with the virus for binding to heparin in the presence of this antibody.

Despite their potential lack of involvement in HCV virion attachment to HS, isolated HCV envelope glycoproteins are able to interact with heparin. Indeed, we confirm that isolated HCV envelope glycoproteins expressed in the HCVcc system are able to interact with heparin. Although this might look in contradiction with the lack of involvement in HCV virion attachment to HS, it is very likely that some HCV glycoprotein regions not exposed at the

surface of the virion could have a heparin-binding motif. We can indeed expect that positively charged amino acids on the non-exposed face of HCV glycoprotein complex interact with the phospholipid head of the virion envelope and could have the capacity to interact with heparin after detergent dissociation. The structure of the core of E2 envelope glycoprotein does not show any extended area of positively charged residues (Khan et al., 2014; Kong et al., 2013). However, one cannot exclude the presence of such a region overlapping E1 and E2.

ApoE is the virion component mediating interaction with HS. This is in agreement with previously published data showing that viruses produced in cells expressing defective heparin binding apoE mutants are not infectious (Jiang et al., 2012). Indeed our inhibition data of virion binding to heparin beads are in agreement with the lack of virus binding to heparin after ablation of the heparin binding sequence of apoE. Furthermore, our kinetic analysis of HCV entry in the presence of anti-apoE antibody and the capacity of the anti-apoE to inhibit virion binding to cells are other arguments supporting the role of apoE in binding to HS.

Binding of viral or cellular ligands to HS depends on defined patterns and orientations of the sulfo and carboxyl groups along the polysaccharide chain (Munoz and Linhardt, 2004). In the liver, HS resemble heparin with a high level of sulfation (1.34 sulfate/ disaccharide, approximately twice the sulfation level observed in other tissues). More specifically, liver HS are rich in *N*-sulfated glucosamine and 2-*O*-sulfated iduronic acid, and contain a high proportion of trisulfated disaccharides (Lyon, Deakin, and Gallagher, 1994). These modifications are not equally spread along the glycosaminoglycan chains, but the highly sulfated motifs are clustered at the extremity of the chain distal from the core protein. The modifications of the HS chains require the action of specific sets of enzyme families. In this work, we analyzed the expression profile of the enzymes involved in the different reactions of sulfation in the Huh-7 hepatoma cell line. It was reported that NDST1 and NDST2 are expressed in the liver, however in Huh-7 cells, NDST2 expression was very low, just above the detection limit. The specific functions of both enzymes are not completely understood. In mice, the knockout of NDST2 does not affect the structure of liver HS, whereas the knockout of NDST1 decreases *N*-sulfation level by 50% (Ledin et al., 2004; Ringvall et al., 2000). The residual *N*-sulfation was attributed to NDST2 suggesting a compensatory effect of NDST2 in the absence of NDST1 expression. It is generally admitted that the reaction catalyzed by NDST is a prerequisite to further modifications. As expected the knockout of NDST1 decreases the level of 2-*O*-sulfation, however in hepatocytes, the level of 6-*O*-sulfated HS is barely affected. That can explain why the silencing of sulfotransferases was not as efficient as that of CD81 to inhibit HCV infection in Huh-7 cells.

Indeed, we could expect that *N*-sulfation was not altered by the silencing of 6-OST1/2, while inhibition of the expression of NDST1 only reduced partially the level of 6-*O*-sulfate groups in HS chains. In line with these data, we observed an increase in the expression level of 6-OST1 when the expression of NDST1 was inhibited with a specific small interfering RNA (data not shown). Interestingly, we provide evidence that the reactions of HS sulfation catalyzed by NDST1 and 6-OST1/2, but not 2-OST, are required for HCV-HS interaction. These findings support the conclusion that specific sulfate groups on cellular HS rather than the total level of sulfation may be important for mediating HCV-host cell interaction. Besides the involvement of *N*- and 6-*O*-sulfate groups, the size of the saccharide chain appears to play an important role in efficient HCV-HS binding. Indeed, marked inhibition of HCV binding to target cells was observed for a dp10 oligosaccharide. These findings indicate that the interaction of hepatitis C virus with highly sulfated HS on target cells is not simply the result of charge interactions but requires a specific HS structure.

In conclusion, despite their capacity to interact with HS after dissociation from the virion with detergent, HCV envelope glycoproteins associated with the viral particle do not expose a HS-binding motif. In contrast, apoE associated with HCV virion is responsible for HCV binding to specific HS involving *N*- and 6-*O*-sulfate groups and a minimum size of a decasaccharide, indicating that HCV hijacks apoE to initiate its interaction with specific HS structures.

ACKNOWLEGEMENTS

This work was supported by the French National Agency for Research on AIDS and Viral Hepatitis (ANRS). Y.X. was supported by a fellowship from the China Scholarship Council (CSC, N° 2011610005) and S.B. by a Marie Curie International Reintegration Grant (PIRG-GA-2009-256300).

We are grateful to Yves Rouillé and Czeslaw Wychowski for their scientific advice. We are grateful to Sophana Ung for his assistance in the illustrations. We also thank M. Law, J. McKeating, S Foung and T. Wakita for providing us with reagents. The fluorescent microscopy data were generated with the help of the Bio-imaging Center Lille Nord-de-France (BICeL).

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FIGURE LEGENDS

Figure 1. HVR1 deleted virus relies on HS for attachment.

(A) Huh-7 cells were inoculated with purified JFH1 or JFH1-ΔHVR1 viruses at a MOI of 5 at 4°C for 2h. Then, the cells were washed with PBS to remove unbound viruses and lysed to extract RNAs. HCV genomes were measured by quantitative RT-PCR. Results are expressed as the mean of three independent experiments in duplicate and error bars represent the standard errors of the mean. Statistical comparisons were made with the two-tailded unpaired Student's t-test.

(B) Purified viruses were pre-incubated with increasing concentrations of heparin for 1h at 37°C, then virus/heparin mixes were chilled and incubated with Huh7 cells at 4 °C for 2h. The inoculum was removed and the cells were washed and incubated for another 30h at 37°C. Infected cells were quantified by indirect immunofluorescence with anti-E1 Mab A4. Results are expressed as the percentage of the control infection without heparin and represent the means of 3 independent experiments in duplicate .

Figure 2. Interaction between heparin and HCV envelope proteins. Lysates obtained from cells infected (A) with JFH1 or JFH1-ΔHVR1 or purified viruses (B) were precipitated with heparin-conjugated beads. Proteins were eluted with Laemmli buffer and samples were separated on SDS-PAGE. HCV envelope proteins were detected by immunoblotting with A4 (anti-E1) and A11 (anti-E2) antibodies. Ctrl represents protein samples from non-infected Huh7 cells.

Figure 3. Anti-ApoE antibodies neutralization JFH1-wt and JFH1-ΔHVR1 viruses were pre-incubated with either control or the polyclonal anti-apoE antibody (dilution 1/500) at 37°C for 1h, then added to the cells for 2h. The inoculum was removed and the cells were washed and incubated for another 30h. Infected cells were detected by indirect immunofluorescence. Results are expressed as the percentage of HCVcc infectivity in the absence of antibodies. Mean values of three independent experiments in duplicate are given. Error bars represent the standard errors of the means. Statistical analysis were made with one-way ANOVA Turkey's Multiple Comparison T test, ***$P < 0.001$.

Figure 4. Anti-apoE antibodies inhibit HCV binding to cell surface. (A) Kinetics of HCV entry inhibition by heparin or anti-apoE antibodies. Infection of the cells with purified JFH1 was

divided in three steps. The virus was inoculated to Huh-7 cells at 4 °C for 1h. Then, the cells were rinsed and incubated at 4 °C for an additional hour. Subsequently, the cells were washed and the temperature was shifted to 37°C for an additional hour. For each step, the presence of 500 µg/ml of heparin or 5µg/ml of anti-apoE Mab 23 is depicted under the x axis. 30h later, cells were fixed and processed for immunofluorescence. Results are expressed as the percentage of control infections without inhibitors. Mean values of three independent experiments are given. Error bars represent the standard errors of the means. Statistical comparisons were made with the two-way RM ANOVA Bonferroni post-tests, ***P < 0.001. (B) Cells were inoculated at 4 °C for 2h with purified virus with or without heparin (500µg/ml) or anti-apoE Mab 23 (5µg/ml). Cells were washed and the virus bound to cell surface was measured by quantitative RT-qPCR. Mean values of three independent experiments in duplicate are given. Error bars represent the standard errors of the means. Statistical comparisons were made with the two-tailded unpaired Student's t-test, *P < 0.05.

Figure 5. ApoE mediates HCV binding to HS. Purified H77/JFH1 chimera was pre-incubated for 1h at 37°C with 25 µg/ml heparin, antibodies (5 µg/ml Mabs AR3A, 10 µg/ml Mabs AR5A, 10 µg/ml Mabs RO4, 20 µl Mabs 9/27, 1/200 anti-apoE polyclonal antibodies or anti-human IgG polyclonal antibodies) or 20 µg/ml apoE-derived peptides before heparin-coated beads were added. Virus bound to the beads was measured in quantitative RT-PCR. The quantity of virus bound to the beads in the absence of any competitor was arbitrary set at 100%. Mean values of three independent experiments in duplicate are given. Statistical comparisons were made with the one-way ANOVA Dunnett's Multiple Comparison T test, ***P < 0.001.

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Figure 7. HS biosynthetic enzymes expression profile. Total RNAs were extracted from Huh-7 cells and mRNA levels of enzymes involved in HS sulfation were quantified by quantitative RT-PCR. Mean values of 3 independent experiments are given. Error bars represent the standard errors of the means.

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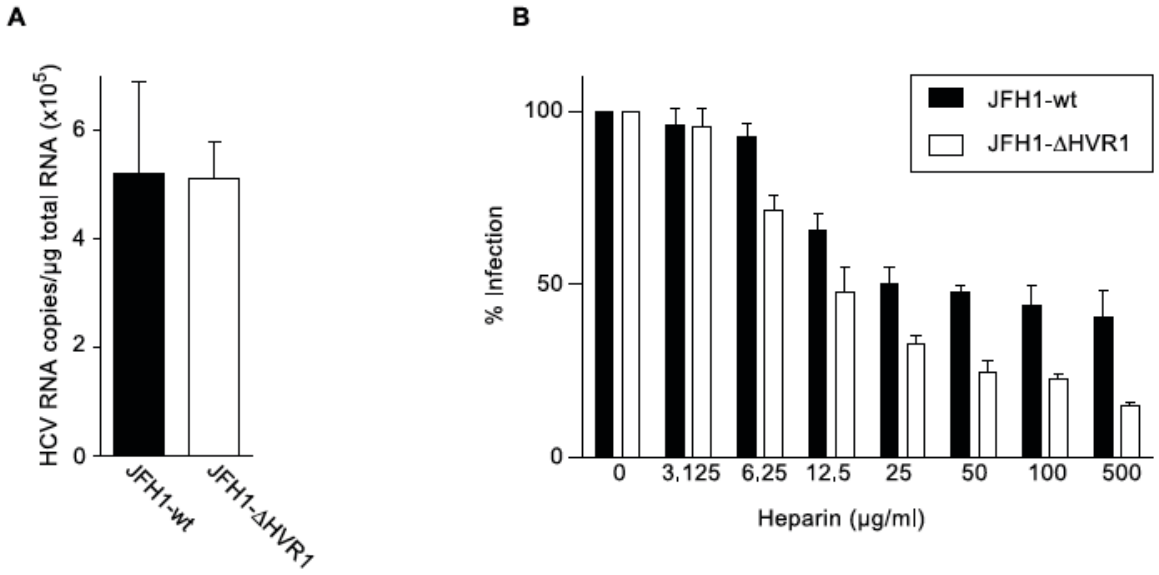

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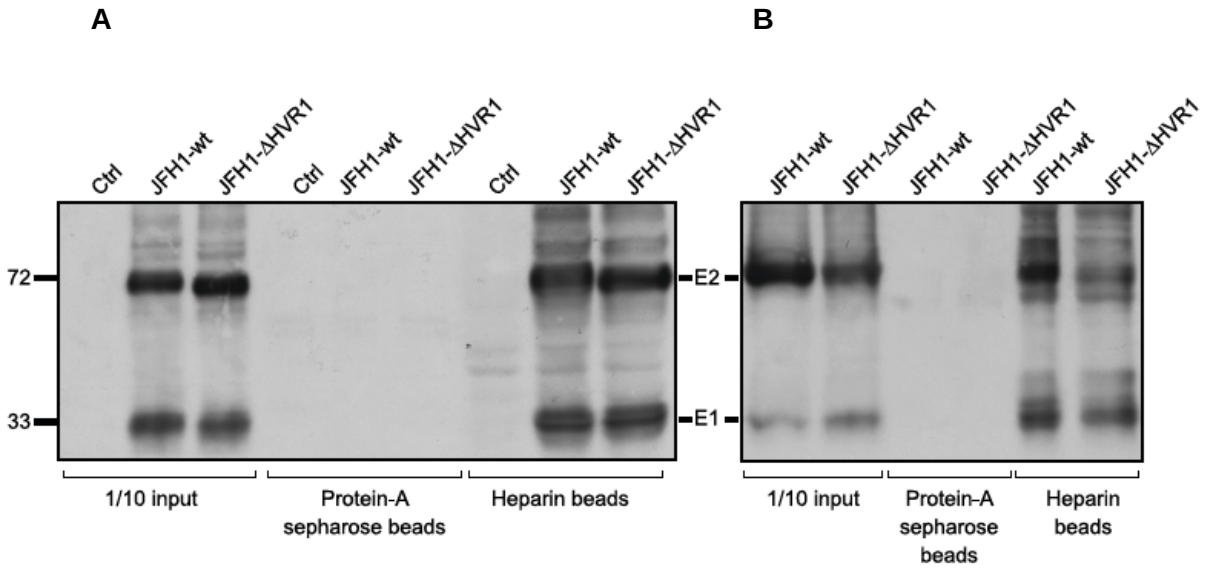

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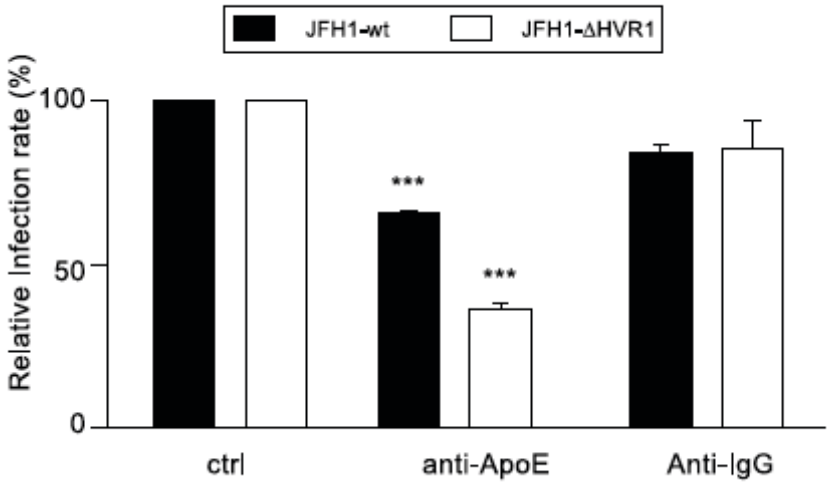

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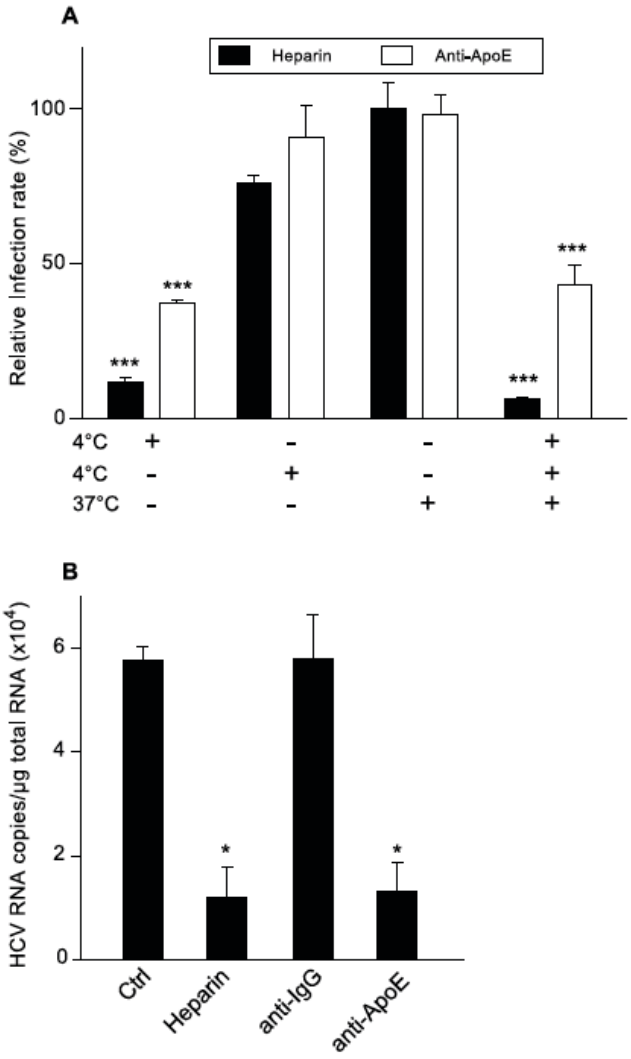

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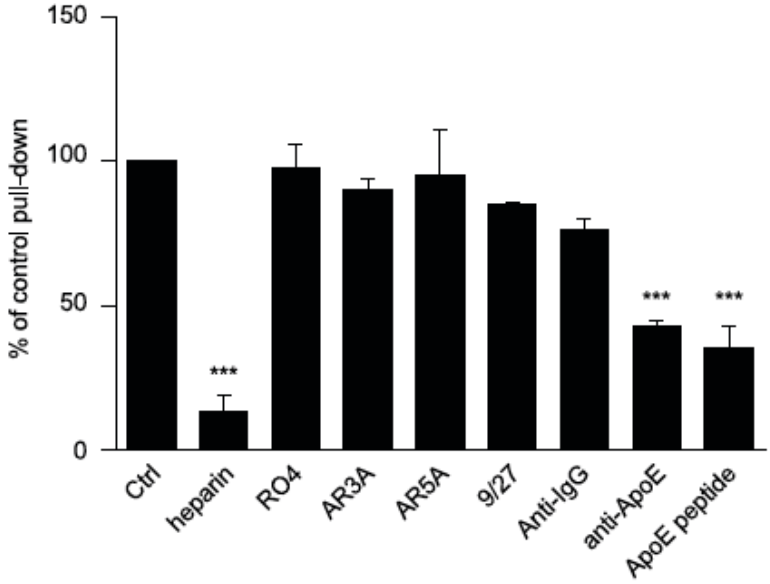

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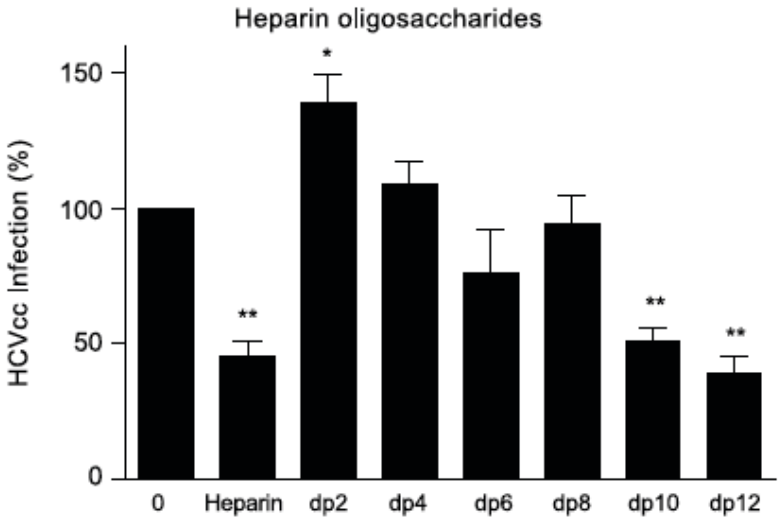

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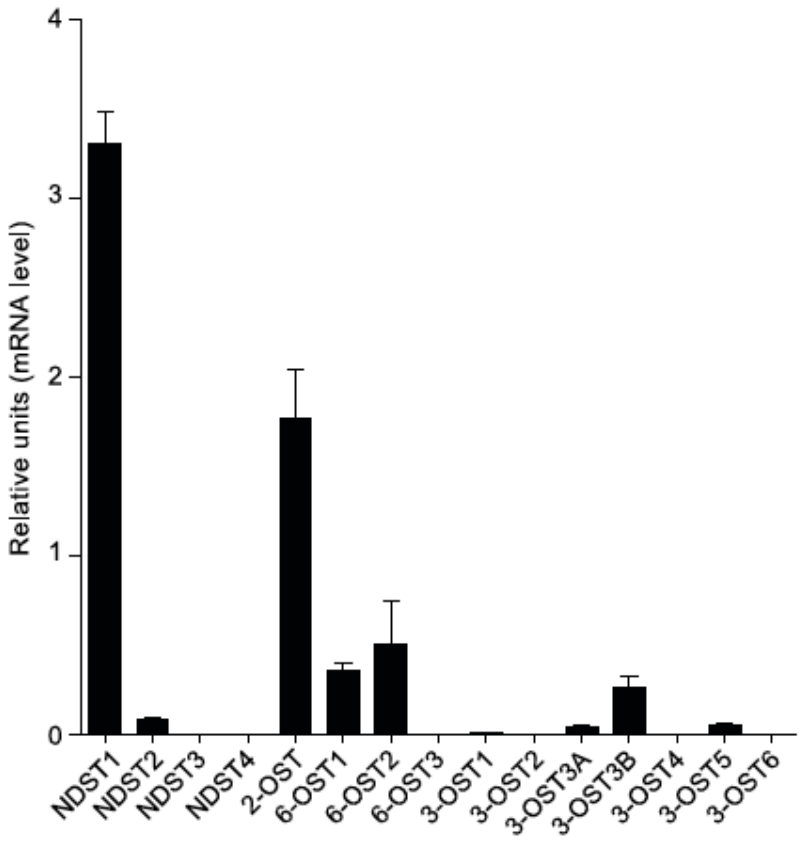

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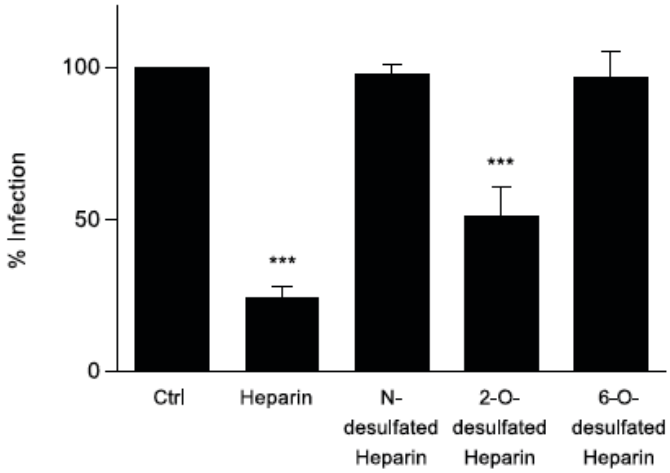

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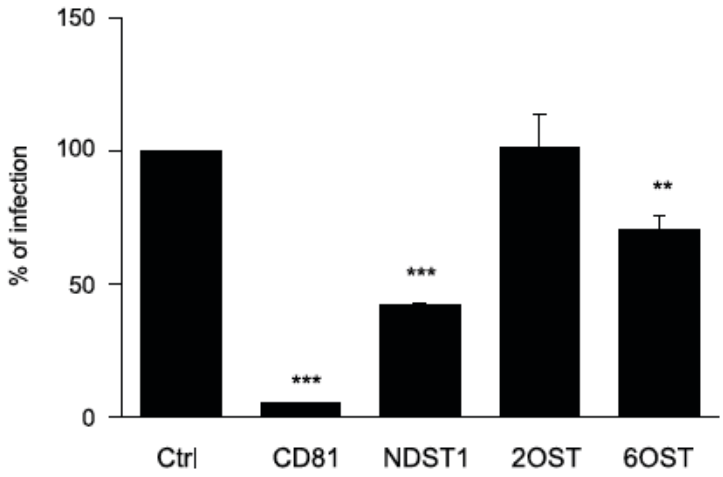

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DISCUSSION & PERSPECTIVES

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I. Virion determinant responsible for HCV binding to cell-surface HS

HS are involved in various biological processes. This explains why HS-binding sites are found in a wide range of proteins that interact with heparin/HS, including enzymes, growth factors, cytokines and chemokines (Handel *et al*., 2005; Ori *et al*., 2008). Many microorganisms, including viruses, often take advantage of the presence of HSPGs exposed at cellular membranes to initiate attachment and infection of targets cells. For instance, bovine viral diarrhoea virus (BVDV), which, like HCV, is also from the *Flaviviridae* family, interacts with highly sulfated HS located on cell surface to initiate its life cycle. Interestingly, a cluster of basic amino acids near the C-terminus of its envelope protein E^{rns} was found to mediate the HS-binding, constituting the HS-binding site (Iqbal & McCauley, 2002). In the case of HCV, studies to characterize the potential involvement of HS in virus entry have been initiated before the development of a cell culture system for this virus. Therefore, the first investigations on the potential role of HS in HCV entry used surrogate models to study HCV-HS interactions. These models included recombinant envelope glycoproteins, Virus-like particles produced in insect cells and HCVpp (Bath *et al*., 2003; Barth *et al*., 2006). These studies were based on the assumption that, as observed for other viruses, envelope proteins present at the surface of the virion should contain HS-binding site (s) if the virus needs HS initiate its life cycle.

In this context, it was reported that recombinant soluble E2 molecule without the N-terminal HVR1 (384-410aa) showed reduced binding to heparin in surface plasmon resonance (SPR) analyses. This observation indicated that the HVR1 of E2 plays a major role in E2 interaction with heparin/HS (Barth *et al*., 2003). In support of this observation, monoclonal antibody 2F10, which targets the middle of HVR1 (398-403aa), significantly inhibited E2 binding to the immobilized heparin. Furthermore, monoclonal antibody AP33 recognizing an epitope close to HVR1 (aa412-423) also displayed a marked inhibition in E2-HS interaction. The HS-binding sites within HCV envelope glycoprotein E2 has also been analyzed by using a set of overlapping peptides corresponding to conserved regions of envelope proteins in solid phase heparin-binding assays. Peptides covering the N-terminal sequence region 491-501aa and

another region 628-638aa displayed the strongest binding, suggesting that these regions in E2 could be involved in HS-interaction (Olenina *et al*., 2005). Furthermore, two additional HS-binding sites within E2-CD81 binding regions were also identified through functional mapping. Monoclonal antibodies 11F11 and 49F3, respectively targeting $E2_{\mathbf{aa480\text{-}487}}$ and $E2_{aa516\text{-}530}$ strongly inhibited E2-HS interaction, suggesting that these regions of E2 may interact with both CD81 and HS (Barth *et al*., 2006). Finally, recombinant E1 protein was also demonstrated to bind HS, suggesting that both envelope glycoproteins of HCV could be involved in this initial interaction (Barth *et al*., 2006).

Heparin-binding motifs contain clusters of basic amino acids such as lysine and arginine. However, not all the HS-binding proteins contain such a conserved linear pattern of amino acids (Margalit *et al*., 1993). In HCV envelope glycoproteins, a large number of lysine, arginine and histidine residues are found to be scattered across the protein sequences but not clustered, suggesting that to interact with heparin, some of these positively charged residues need to be reorganized in the context of the 3D structure of the proteins in order to form a heparin binding site. The recently published structure of the core of E2 protein does not clearly show a large patch of positively charged amino acids which could form a heparin-binding motif (Kong *et al*., 2013; Khan *et al*., 2014). However, one cannot exclude that, in the context of the full-length protein and/or in the presence of E1, a heparin-binding binding site could be formed. Alternatively, we cannot exclude that, in the context of our experiments with lysates of particles or infected cells, a cellular partner of HCV envelope glycoproteins could be responsible for the interaction with heparin. However, this hypothesis does not fit with the observation that purified recombinant HCV envelope glycoproteins interact with heparin (Barth *et al*., 2003; Barth *et al*., 2006).

In our experiments, we worked with HCV envelope glycoproteins produced in the context of infected cells or associated with purified virions. In this context, we showed that HCV envelope glycoproteins also bound to immobilized heparin, in agreement with data obtained with recombinant proteins. However, HCV envelope glycoproteins isolated from cells infected by HVR1-deleted virus or purified HVR1-deleted viral particles, were still able to bind heparin efficiently, indicating that HVR1 does not play a major role in this interaction. A major difference between our study and previous reports is that we worked with E1E2 complexes instead of isolated E2 or E1 glycoprotein. Therefore, one cannot exclude that, even in the absence of HVR1, determinants present in E1 and E2 can lead to a strong interaction

with heparin, whereas in the context of recombinant E2 protein, the lack of HVR1 would reduce the affinity for heparin in the absence of E1.

HCV envelope glycoproteins do not seem to play a role in the initial interaction of HCV particles with HS. We first concentrated our study on HVR1, which is supposed to be the most exposed region of HCV envelope glycoproteins and because it had been proposed to be involved in interactions with HS. However, contrary to what has been previously reported, HVR1 sequence of E2 glycoprotein does not seem to be involved in the binding of HCV particle to HS. In our work, in addition to biochemical experiments discussed above, we also used a functional approach to reinvestigate the potential contribution of HVR1 to HCV glycoproteins interaction with HS. However, our functional experiments did not confirm a role for HVR1 in this interaction. Previous functional studies reporting the involvement of HVR1 in HCV-HS interaction were made with the help of pseudoparticles (Barth *et al*., 2006; Basu *et al*., 2007). However, these model systems are not the most appropriate tools to study such interactions since pseudoparticles devoid of any viral envelope protein can directly bind to target cells (Pizzato *et al*., 1999), and such envelope-independent binding can be inhibited by heparin (Walker *et al*., 2002), indicating that non viral proteins incoporated in such particles are responsible for the binding of these particles to HSPGs. Although other experiments performed with the HCVcc system also suggested a role for HVR1 in HCV binding to HS (Koutsoudakis *et al*., 2012), the authors only performed functional studies without biochemical confirmation, and the phenotype observed could be due to indirect effects of the mutations, since HVR1 is involved in interaction with SRB1 (Scarselli et al., 2002).

We further investigated the potential involvement of HCV envelope glycoproteins in virion binding to HS. However, the protein surface of HCV envelope glycoproteins is poorly exposed at the surface of viral particles which is due in part to the association with lipoproteins (Merz *et al*., 2011) and to the presence of a large number of *N*-linked glycans (Helle *et al*., 2010; Kong *et al*., 2013). These features dramatically reduce the possibilities for these glycoproteins to interact with HS when associated with the viral particle. They also explain the limited number of neutralizing epitopes identified on HCV glycoproteins. However, we used antibodies recognizing different regions of HCV envelope glycoproteins to probe the potential interaction of HCV envelope glycoproteins for their interaction with HS in the context of the virion. As shown with Mab AR3A, the CD81-binding region is not

involved in HCV-HS interaction, which is in agreement with a similar observation made with another Mab targeting this region (Jiang *et al*., 2012). HVR1 is the most virion-exposed region of HCV envelope glycoproteins, and in agreement with the data obtained with our HVR1-deletion mutant, Mab 9/27, recognizing an epitope in this region (Hsu *et al*., 2003), did not inhibit HCV-HS interaction. Finally, we also used a neutralizing antibody recognizing an epitope shared by E1 and E2 and located outside of the CD81-binding region (Giang *et al*., 2012), and again no competition was observed with the virus for binding to heparin in the presence of this antibody.

Since HCV envelope glycoproteins do not seem to be involved in HCV virion interaction with HSPGs, other components of the viral particle must be involved in this initial binding since functional studies have shown that HSPGs play a role in HCV entry. It is important to note that the HCV life cycle, particularly virus assembly and production, is closely linked to cellular lipid metabolism (Huang *et al*., 2007), which results in a unique feature of HCV that cellular apolipoproteins are associated with viral particles (Andre *et al*., 2002; Nielsen *et al*., 2008; Shimizu *et al*., 2011). By being present on the surface of the virions, these apolipoproteins could play some role (s) in HCV entry though interacting with cellular entry factors present at the surface of hepatocytes. It is now well demonstrated that apoE is a major structural component of HCV virion, and content analysis of purified HCVcc showed that each particle bears around 300 molecules of apoE at its surface, suggesting a remarkable enrichment of apoE molecule by HCV virion (Merz *et al*., 2010). Consistently, in the study of Catanese et al, analyzing infectious purified particles through virion immunocapture, double–immunolabeling and cryoelectrontomography (cryo-ET) studies, data showed that host-derived apoE is better exposed on HCV envelope than viral glycoproteins (Catanese *et al*., 2013b). Since apoE is known to contain HS-binding motifs, it has been proposed that this apolipoprotein could be the viral component responsible for virion binding to HSPGs. Indeed, previous studies have shown that apoE has at least two heparin-binding sites located in the N- and C-terminal regions of this protein (Saito *et al*., 2003a; Saito *et al*., 2003b; Yamauchi *et al*., 2008). However, the C-terminal motif is not accessible for heparin binding in both the lipid-free and lipidated states of the intact apoE molecule, indicating that only the N-terminal motif contributes to the interaction of apoE with HSPGs *in vivo* (Dong *et al*., 2001; Libeu *et al*., 2001; Saito *et al*., 2003b).

In our work, we confirmed the important role of apoE in mediating HCV attachment and entry by binding to cell surface HS. Our data is consistent with the study of Jiang *et al.*, which demonstrated that HSPGs serve as a major HCV attachment factor, and that apoE on the viral envelope mediates HCV attachment by binding to cell-surface HSPGs (Jiang *et al*., 2012; Jiang *et al*., 2013). Indeed our inhibition data of virion binding to heparin beads are in agreement with the lack of virus binding to heparin after deletion of the heparin binding sequence of apoE. Furthermore, our kinetic analysis of HCV entry in the presence of anti-apoE antibody and the capacity of the anti-apoE to inhibit virion binding to cells are other arguments supporting the role of apoE in binding to HS. Altogether, our results support the hypothesis that the virion component apoE but not HCV envelope glycoproteins mediate HCV cell binding by interacting with HS.

II. Structural determinant of HS required for HCV infection

In the liver, HSPGs resemble heparin with a high level of sulfation (1.34 sulfate/ disaccharide, approximately twice the sulfation level observed in other tissues). More specifically, liver HS are rich in *N*-sulfated glucosamine and 2-*O*-sulfated iduronic acid, and contain a high proportion of trisulfated disaccharides (Lyon & Gallagher, 1994). These modifications are not equally spread along the glycosaminoglycan chains, but the highly sulfated motifs are clustered at the extremity of the chain distal from the core protein. Importantly, the modifications of the HS chains require the action of specific sets of enzyme families. Under normal physiological condition, hepatic HSPGs play a crucial role in the clearance of apoE-rich TRL particles (AI-Haideri *et al*., 1997), which is mediated by multivalent binding of HSPG to apoE and apoAVI on the surface of lipoprotein particles (Gonzales *et al*., 2013). It was found that each VLDL particle carries 5-7 apoE molecules (Tomiyasu *et al*., 2001). The specific apoE-binding sequence in heparin/heparan sulfates was previously suggested to be highly sulfated (Bazin *et al*., 2002). Investigations in NDST1 liver-specifically knock-out mice showed that the liver HS composition was dramatically changed. NDST1-deleted hepatocytes displayed a defective clearance of TRL-rich lipoproteins, suggesting that the N-sulfation of HS is very important for its function (MacArthur *et al*., 2007). Similarly, study in 2-OST knock-out mice also displayed high lipoprotein level in the blood. Furthermore, chemically modified heparins lacking N-sulfate and 2-O-sulfate group were not able to block

VLDL binding and uptake by isolated hepatocytes, suggesting the 2-O-sulfates of HS is required for TRL clearance (Stanford *et al.*, 2010).

To address the HS structural determinants important for interaction with HCV, we first analyzed the minimal HS length unit required to inhibit HCV infection by using heparin-derived oligosaccharides of a defined length. We observed a 50% decrease of infection in the presence of heparin, and pre-incubation of the virus with oligosaccharides of dp10 or dp12 also inhibited HCV infection at levels similar to heparin. These data indicate that a minimum of 10 saccharides is necessary for HCV virion interaction with HS. These data could provide novel approaches for developing anti-HCV therapies targeting HCV entry or other viral infections. Indeed, a growing number of studies have shown that HS and its derivatives and analogues can inhibit viral entry of many different viruses including HIV-1 and HSV (Hu *et al.*, 2011; Rudd *et al.*, 2012).

In our work, we analyzed the expression profile of the enzymes involved in the different reactions of sulfation in the Huh-7 hepatoma cell line in order to further characterize HS determinants involved in HCV binding. HS biosynthesis is divided in 3 main steps: chain initiation, polymerization and modification. The initiation step is characterized by the linkage of a tetrasaccharide to the proteoglycan core protein. Then, HS backbone is formed by the assembly of alternating glucuronate (GlcUA) and *N*-acetylglucosamine (GlcNAc) residues. As the chain assembles, it undergoes a series of modifications catalyzed by a C_5 epimerase and multiple sulfotransferases. The first modification of the chain to occur is the removal of *N*-acetyl group from subsets of GlcNAc and the addition of *N*-sulfo group. This reaction is orchestrated by members of the NDST family. Further modifications of HS include epimerization of some glucuronate to iduronate residues, addition of sulfate groups at C_2 of uronic acid by 2-OST, and at C_6 and/or at C_3 of GlcN residues by 6-OST and 3-OST, respectively. Although 2-OST is represented by a unique isoform, 6-*O*-sulfation and 3-*O*-sulfation of HS can be catalyzed by three 6-OSTs and seven 3-OSTs.

Our data indicate that the expression of NDST2 is very low in Huh-7 cells, while NDST3 and NDST4 transcripts were not detected. We also found a high level of 2-OST transcripts and a modest expression of 6-OST1 and 6-OST2, both isoenzymes being involved in the same 6-*O*-sulfation reaction. Furthermore, we only detected very low levels of 3-OST enzymes involved in the 3-*O*-sulfation reaction. It was reported that NDST1 and NDST2 are expressed in the liver, however in Huh-7 cells, NDST2 expression was very low, just above the detection limit.

The specific functions of both enzymes are not completely understood. In mice, the knockout of NDST2 does not affect the structure of liver HS, whereas the knockout of NDST1 decreases *N*-sulfation level by 50% (Ringvall *et al*., 2000; Ledin *et al*., 2004). The residual *N*-sulfation was attributed to NDST2 suggesting a compensatory effect of NDST2 in the absence of NDST1 expression. It is generally admitted that the reaction catalyzed by NDST is a prerequisite to further modifications. As expected the knockout of NDST1 decreases the level of 2-*O*-sulfation, however in hepatocytes, the level of 6-*O*-sulfated HS is barely affected. This can explain why the silencing of sulfotransferases was not as efficient as that of CD81 to inhibit HCV infection in Huh-7 cells.

To determine if a particular pattern of sulfation is important for HCV-HS interactions, we performed infection in the presence of chemically modified heparins. Interestingly, the 2-*O*-desulfated heparin was able to inhibit HCV infection by 50% whereas unmodified heparin inhibits 80% of HCV infection. In contrast, *N*- or 6-*O*-desulfated heparins used at the same concentrations were unable to inhibit HCV infection. These results suggest that *N*- and 6-*O*-sulfo groups are important for HCV infection. These experiments were completed with the knock down of some enzymes involved in these modifications. Interestingly, we observed a 60% decrease of HCV infection in cells silenced for NDST1, indicating that *N*-sulfation is necessary for HCV interaction with GAGs. HCV infection was unaffected by the silencing of 2-OST, whereas the silencing of both isoforms of 6-OST reduced HCV infection. These data are in agreement with the results obtained with the desulfated heparins and support the idea that *N*- and 6-*O*-sulfation but not 2-*O*-sulfation are required for HCV infection. A previous report indicated that HS-binding of HCVpp, which lack apolipoproteins, need both the 2-O-sulfo- and the 6-O-sulfo groups for binding to cell-surface, and that, HS-binding of recombinant E1 and E2 proteins were also found to require the 2-O-sulfo and 6-O-sulfo groups (Barth *et al*., 2006). However, as discussed above, the HCVpp system is not the most appropriate tool to study HS-virion interactions since pseudoparticles devoid of any viral envelope protein can directly bind to target cells (Pizzato *et al*., 1999), and such envelope-independent binding can be inhibited by heparin (Walker et al., 2002). indicating that non viral proteins incoporated in such particles are responsible for the binding of these particles to HSPGs. Furthermore, the fact that data obtained with recombinant envelope glycoproteins do not reflect those obtained with infectious virus are in agreement with our study which suggests that HCV envelope glycoproteins are not directly involved in HCV virion interaction with HSPGs. Interestingly, our data are in contrast to the observation which indicated that 2-

O-sulfates of HS are required for VLDL binding to HSPGs (Stanford *et al*., 2010). This suggests that, despite its interaction with lipoprotein components, HCV particle does not behave like a VLDL for its initial binding to hepatocytes. This is in line with the observation that by simply expressing apoE in 293T cells transfected with HCV, infectious viral particles can be produced (Da Costa *et al*., 2012; Hueging *et al*., 2013), indicating that HCV virions are not similar to VLDLs which also contain apoB and probably other lipoprotein.

Heparin-binding and/or anti-heparan sulfate peptides could be interesting for the development of novel potential antiviral molecules against HCV, since a number of peptides were found to inhibit various virus attachment by binding to cell-surface HSPGs (Luganini *et al*., 2010; Tiwari *et al*., 2011). For instance, linear heparin-binding peptides derived from the fusion glycoprotein of human respiratory syncytial virus (RSV) are capable of inhibiting the infection of some RSV strains (Crim *et al*., 2007). In our study, human apoE-derived heparin-binding peptide inhibited HCV virion-heparin binding. We did not test its effect on HCV infection in a functional study, but another human apoE peptide, hEP which contains both the receptor-binding and lipid-binding fragments was demonstrated to block HCVcc entry at submicromolar concentrations (Liu *et al*., 2012).

Together, our data provide evidence that the reactions of HS sulfation catalyzed by NDST1 and 6-OST1/2, but not 2-OST, are required for HCV-HS interaction. These findings support the conclusion that specific sulfate groups on cellular HS rather than the total level of sulfation may be important for mediating HCV-host cell interaction. Besides the involvement of *N*- and 6-*O*-sulfate groups, the size of the saccharide chain appears to play an important role in efficient HCV-HS binding. Indeed, marked inhibition of HCV binding to target cells was observed for a dp10 oligosaccharide. These findings indicate that the interaction of HCV with highly sulfated HS on target cells is not simply the result of charge interactions but requires a specific HS structure. Finally, our study also provides knowledge on novel potential targets for anti-HCV intervention.

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